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Exploring the toxicological and physiological functions of the AH receptor

Selective modulation by two novel compounds and involvement in novel food avoidance behaviour

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ACADEMIC DISSERTATION

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ABSTRACT

The aim of this thesis study was to gain more information on the physiological and toxicological functions of the aryl hydrocarbon receptor (AHR). The effects of two novel selective AHR modulators (SAHRMs) were investigated *in vitro* and *in vivo*. In addition, the involvement of the AHR in the avoidance of novel food was examined.

The AHR is an evolutionarily ancient, apparently over 600-million-year-old protein. It is a ligand-activated transcription factor that modulates the expression of various genes within cells. One of the most studied groups of compounds that activate the AHR are dioxins. They are environmental contaminants primarily formed as by-products of various industrial processes, and many of them are toxic. Dioxins are chemically very persistent and lipid soluble, and thus accumulate in the food chain. Therefore, humans are also exposed to small amounts from food. In Finland, the most common source of dioxins is fatty wild fish from the Baltic Sea.

The AHR has been recognised as the mediator of dioxin-induced toxicity for decades. More recently, it has also been shown to be involved in several physiological functions of the body, including the regulation of reproduction, foetal development, the immune system and autoimmunity. However, our understanding of the mechanisms of both the toxicological and physiological functions of the AHR remains incomplete. As a consequence, for instance, human health risk assessment of dioxins is challenging. Furthermore, better understanding of the physiological effects of the AHR could help elucidate the aetiology and pathogenesis of certain diseases, and therefore also benefit the discovery of novel pharmacological therapies.

As lead compounds for drug discovery, SAHRMs are particularly interesting. They only elicit subsets of AHR-mediated effects, often without the major toxic outcomes of dioxins. Moreover, they could be valuable tools in elucidating the so far incompetently understood, multifaceted physiological roles of AHR, and the underlying molecular mechanisms.

This thesis research had two main objectives. The first was related to studying the *in vitro* and *in vivo* toxicity of two novel SAHRMs, which are intended as drug compounds for the treatment of autoimmune diseases. The aim was to determine whether they appear suitable for pharmacological use from the pre-clinical safety perspective. Furthermore, finding out the extent to which their effects resemble or differ from those of the most toxic dioxin, TCDD, was of interest. The second objective was to accumulate more knowledge on a peculiar novel food avoidance behaviour, previously characterised in rats and mice after exposure to TCDD. This behaviour resembles a recognised behaviour model, conditioned taste aversion (CTA), which is also exhibited in humans, for instance in conjunction with nausea related to cancer treatment. The aim here was to verify whether the aforementioned rodent response

is a physiological effect of the AHR or, more specifically, a consequence of TCDD exposure.

Based on the results, the novel SAHRMs are very effective AHR activators, both *in vitro* and *in vivo*, and are in fact comparable to TCDD. However, their toxicity profiles are distinct from that of TCDD, and they appear considerably less toxic in rats. Therefore, the novel SAHRMs appear promising as possible drug compounds, and also highly interesting as tools for AHR research. Despite the differences in toxicity, one of the novel SAHRMs, as well as all of the three other AHR activators tested in this study, induced a strong avoidance response resembling that previously observed to TCDD, but shorter lasting. In addition, the reaction was not inducible in AHR knock-out rats. Thus, this study confirmed that the novel food avoidance behaviour is mediated by the AHR. The effect appears protective against potentially harmful ingested foods, and is therefore another physiological function of the AHR.

TIIVISTELMÄ

Tämän väitöskirjatutkimuksen tarkoituksena oli saada lisätietoa aryylihiilivetyreseptorin (AHR) fysiologisista vaikutuksista elimistössä sekä sen kautta välittyvän toksisuuden mekanismeista. Tutkimme kahden uuden, valikoivasti AHR:n toimintaa säätelevän lääkeainekandidaatin vaikutuksia. Lisäksi tarkastelimme AHR:n osallisuutta aiemmin kuvatussa, uusien ruoka-aineiden karttamisreaktiossa.

AHR on evolutiivisesti muinainen, ilmeisesti yli 600 miljoonaa vuotta vanha proteiini, joka säätelee soluissa lukuisten geenien ilmentymistä. Yksi eniten tutkituista AHR:n toimintaa aktivoivista aineryhmistä on dioksiinit. Ne ovat ympäristölle ja terveydelle haitallisia yhdisteitä, joita syntyy pääasiassa lämpö- ja teollisuusprosessien sivutuotteina. Koska ne ovat kemiallisesti erittäin pysyviä ja hyvin rasvaliukoisia, ne kertyvät ravintoketjuissa, ja siten myös ihmis*et al*tistuvat niille ravinnon välityksellä. Suomessa tavallisin dioksiinien lähde on Itämeren rasvainen villikala.

AHR on tunnettu dioksiinien aiheuttamien myrkyllisten vaikutusten välittäjänä jo pitkään. Sittemmin sillä on osoitettu olevan myös lukuisia elimistön normaalin toiminnan kannalta tärkeitä fysiologisia tehtäviä, muun muassa lisääntymisen säätelyssä, yksilönkehityksessä, autoimmuniteetissa ja immuunipuolustuksessa. Fysiologisten tehtävien, samoin kuin AHR:n välittämien toksisten vaikutusten mekanismien tuntemus on kuitenkin toistaiseksi puutteellista, mikä muun muassa vaikeuttaa dioksiinien ihmisille aiheuttamien terveysriskien arviointia. AHR:n fysiologisten vaikutusten parempi tuntemus voisi lisäksi auttaa tiettyjen sairauksien syntymekanismien selvittämisessä ja siten edistää uusien lääkehoitojen kehitystä.

Selektiivisesti AHR:n toimintaa säätelevät aineet ovat lääkekehityksen kannalta erityisen kiinnostavia, sillä monet niistä aktivoivat AHR:ää aiheuttamatta dioksiineille tyypillisiä haittavaikutuksia. Lisäksi ne voivat olla hyödyllisiä työkaluja AHR:n fysiologisten vaikutusten tutkimisessa.

Tällä väitöskirjatutkimuksella oli kaksi päätavoitetta. Ensimmäinen liittyi kahden uuden, AHR:ää valikoivasti aktivoivan lääkeainekandidaatin toksikologisten vaikutusten tutkimiseen. Tarkoituksena oli selvittää, vaikuttavatko ne toksisuusprofiilinsa puolesta sopivilta lääkeaineiksi, jolloin niitä voitaisiin edelleen kehittää esimerkiksi autoimmuunisairauksien hoitoon. Lisäksi olimme kiinnostuneita selvittämään, missä määrin näiden aineiden aiheuttamat vaikutukset muistuttavat tai poikkeavat myrkyllisimmän dioksiinin, TCDD:n, aiheuttamista vaikutuksista. Toisena tavoitteena oli syventää tietoa aiemmin rotilla ja hiirillä TCDD-annostelun jälkeen havaitusta uusien ruoka-aineiden karttamisreaktiosta, joka muistuttaa ennestään tunnettua käyttäytymismallia, ehdollistettua makuaversiota (CTA). Samankaltainen makuaversio tunnetaan myös ihmisillä muun

muassa syövän hoitoon liittyvän pahoinvoinnin yhteydessä. Halusimme varmistaa, johtuuko edellä mainittu jyrsijöiden reaktio yleisesti AHR-aktivaatiosta vai onko se ainoastaan TCDD:lle ominainen vaikutus.

Tulosten perusteella edellä mainitut uudet, selektiiviset AHR-modulaattorit ovat tehokkaita, TCDD:n veroisia AHR-aktivaattoreita. Toksisuusprofiililtaan ne ovat kuitenkin olennaisesti TCDD:tä haitattomampia ja vaikuttavat siten kiinnostavilta lääkeainekandidaateilta. Havaituista eroista huolimatta sekä toinen niistä että muut tässä työssä testatut AHR-aktivaattorit aiheuttivat TCDD:n tavoin voimakkaan, joskin lyhytkestoisemman, uusien ruoka-aineiden karttamisreaktion. Lisäksi reaktio puuttui AHR-poistogeenisiltä rotilta. Tulos vahvistaa, että tämä mielenkiintoinen vaikutus on AHR-välitteinen. Tämä käyttäytymismuutos suojaa ilmeisesti eliöitä haitalliselta vaikuttavan ravinnon nauttimiselta ja on siten uusi AHR:n fysiologinen vaikutus.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

I Mahiout, S. and Pohjanvirta, R. Aryl hydrocarbon receptor agonists trigger avoidance of novel food in rats. *Physiology & Behavior*. 2016. 167: 49-59.

II Mahiout, S., Lindén, J., Esteban, J., Sánchez-Pérez, I., Sankari, S., Pettersson, L., Håkansson, H., and Pohjanvirta, R. Toxicological characterisation of two novel selective aryl hydrocarbon receptor modulators in Sprague-Dawley rats. *Toxicology and Applied Pharmacology*. 2017. 326: 54-65.

III Mahiout, S., Giani Tagliabue, S., Nasri, A., Omoruyi, M., Pettersson, L., Bonati, L., and Pohjanvirta, R. *In vitro* toxicity and *in silico* docking analysis of two novel selective AH-receptor modulators. *Submitted.*

ABBREVIATIONS

AHH	Aryl hydrocarbon hydroxylase
AHR	Aryl hydrocarbon receptor (protein)
Ahr	Aryl hydrocarbon receptor (gene encoding the protein)
AHRE	AHR responsive element (also known as DRE and XRE)
AHRKO	AHR knock-out rat line
AHRR	AHR repressor
ALAT	Alanine aminotransferase
ANOVA	Analysis of variance
ARNT	AHR nuclear translocator
ASAT	Aspartate aminotransferase
BaP	Benzo-*a*-pyrene
bHLH–PAS	Basic helix-loop-helix-PER-ARNT-SIM transcription factors
BNF	β-Naphthoflavone
BW	Body weight (kg)
CAR	Constitutive androstane receptor
cDNA	Complementary DNA
CNS	Central nervous system
CS	Conditioned stimulus
CTA	Conditioned taste avoidance; often also conditioned taste aversion
CYP	Cytochrome P450 (mRNA or protein)
Cyp	Cytochrome P450 (gene encoding the protein)
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DRE	Dioxin responsive element
EAE	Experimental autoimmune encephalomyelitis; MS model
EMEM	Eagle's Minimum Essential Medium
EMH	Extramedullary myeloid haematopoiesis
ER	Oestrogen receptor
FBS	Foetal bovine serum
FFA	Free fatty acids
FICZ	6-Formylindolo[3,2-b]carbazole
gDNA	Genomic DNA
Hsp90	90-kDa heat shock protein
H/W	Han/Wistar (*Kuopio*) rat strain; Resistant to lethality of TCDD
I3C	Indole-3-carbinol
ig	Intragastric administration
ip	Intraperitoneal administration
ITE	2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester

KLF6	Krüppel-like factor 6
KO	Knock-out (rat or mice line)
LBD	Ligand binding domain
LD50	Lethal dose 50%
LDH	Lactase dehydrogenase
L-E	Long-Evans (*Turku/AB*) rat strain; Sensitive to TCDD
LiCl	Lithium chloride
LOAEL	Lowest observed adverse effect level
MCDF	6-methyl-1,3,8-trichlorodibenzofuran
mRNA	Messenger RNA
MS	Multiple sclerosis
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa B
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-*p*-dioxin
PCDF	Polychlorinated dibenzofuran
PCR	Polymerase chain reaction
POPs	Persistent organic pollutants
qPCR	Quantitative real-time PCR
RNA	Ribonucleic acid
RT	Reverse transcription
RT-qPCR	Reverse transcription quantitative real-time PCR
SAHRMs	Selective AHR modulators
SD	Sprague Dawley rat strain
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEF	Toxic equivalence factor
TEQ	TCDD equivalent
TiPARP	TCDD-inducible poly (ADP-ribose) polymerase
UGT	UDP-glucuronosyltransferase
US	Unconditioned stimulus
WHO	World Health Organization
WT	Wild-type rat line
XRE	Xenobiotic responsive element

1 INTRODUCTION

Research on the aryl hydrocarbon receptor (AHR) started in the 1970s, when this protein, a ligand-activated transcription factor, was first discovered (Poland *et al.* 1976). Initially, it was found to mediate the induction of xenobiotic metabolising enzymes in response to a large group of environmental contaminants, encompassing halogenated and polycyclic aromatic hydrocarbons, which are mostly by-products of industrial thermal processes and incomplete combustion (Kulkarni *et al.* 2008). Soon, the AHR was also established as the mediator of toxicity triggered by these compounds (Poland and Knutson 1982), of which dioxins (polychlorinated dibenzo-*p*-dioxins, dibenzofurans and co-planar PCBs) are particularly important. This initiated a rapidly emerging field of research, where the various toxic effects induced by dioxins were characterised in several laboratory animal species. The model compound largely used in these studies was the most toxic dioxin congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Gasiewicz and Henry 2011, Okey 2007).

Dioxins are widely present in the environment, and humans are also exposed to small amounts of them, mainly via food (Malisch and Kotz 2014, Travis and Hattemer-Frey 1991). There have additionally been several major accidental industrial releases, exposing populations to large quantities. The toxicological risk assessment of dioxins is based on increasing knowledge of their chemical properties and biological effects. Risk assessment has led to risk management measures, which aim to restrict human exposure and further industrial emissions into the environment. In addition, recommendations for risk groups have been given in order to limit the consumption of foodstuffs with high dioxin levels, such as certain Baltic Sea fish (Finnish Food Safety Authority Evira 2017). It is also now commonplace in many countries to monitor dioxin levels in food and livestock feed to detect cases of accidental contamination. Due to these actions, human exposure to dioxins has been declining (Malisch and Kotz 2014, Scientific Committee on Food 2000).

However, human dietary exposure to dioxins remains close to the European tolerable weekly intake value, which denotes an estimate of the amount of dioxins humans can be exposed to throughout their lives without substantial health risks (Bilau *et al.* 2008, Kiviranta *et al.* 2004, Scientific Committee on Food 2001). Furthermore, dioxins are chemically extremely persistent, and will continue to be present in the environment. They are also highly hydrophobic and thus accumulate in the food chain (H. Geyer *et al.* 1986, H. J. Geyer *et al.* 1986). Therefore humans will be at risk of exposure to them also in the future (Malisch and Kotz 2014). However, our understanding of the adverse outcome pathways leading to dioxin toxicity, as well as of the health hazards they pose for humans in general, remain surprisingly incomplete, despite long-term research efforts and advancements made thus far. This is in large part due to the complexity of AHR functioning. In addition,

there is considerable variation in the effects of dioxins depending on, for instance, the species, strain, sex, age, developmental stage and organ in question (Pohjanvirta and Tuomisto 1994, Poland and Knutson 1982). Therefore, to better understand dioxin effects in humans, the need to increase our understanding of the molecular mechanisms underlying AHR-mediated toxicity remains.

Insight into these mechanisms will also contribute to the study of physiological functions of the AHR, where research interest has more recently expanded. This has been a natural consequence of the evidence that the protein is evolutionarily ancient, apparently over 600 million years old (Hahn and Karchner 2011). Characteristically, such evidence suggests paramount importance in the biology of organisms. Indeed, AHR homologues are widespread across vertebrates, and are found in most of their cell types. They have also been found in invertebrates, but, interestingly, do not appear to bind TCDD in them (Butler *et al.* 2001, Hahn 2002). Therefore, the notion that in addition to mediating dioxin toxicity, the AHR could also have important physiological functions in vertebrates has become apparent. This hypothesis already stands confirmed, and the AHR has been shown to be important in normal development and health, although we are only beginning to discover and understand its varied physiological roles.

One example of a seemingly AHR-related, protective physiological consequence is its involvement in novel food avoidance. This bizarre and intriguing, very sensitive effect has previously been characterised after low, well below acutely toxic doses of TCDD in rats and mice (Lensu *et al.* 2011b, J. T. Tuomisto *et al.* 2000). So far, it remains to be established whether the effect is specific to TCDD alone or a result of more general AHR activation.

Further elucidating the many roles and mechanisms of the AHR in normal physiology as well as in toxicity will not merely be important in order to increase our knowledge and improve the accuracy of human health risk assessment. It will also conceivably aid in understanding the pathogenesis of AHR-related maladies and in the development of novel therapeutics for them. Regarding the latter, selective AHR modulators (SAHRMs) appear particularly interesting lead compounds. They modulate AHR activity by only eliciting a subset of the effects imparted by the receptor, often without causing the marked toxicity of dioxins. SAHRMs also have potential as important research tools in studying the molecular mechanisms of AHR function and are thus under intense scrutiny.

In this thesis research, experimental *in vivo* and *in vitro* models were first employed to characterise the toxicological effects of two such novel SAHRMs. One of these compounds was then used, along with other AHR activators, to further examine the peculiar novel food avoidance behaviour in rats. The hypothesis regarding this effect was that it is not only caused by TCDD, but that the AHR acts as a more general biological sensor of the environment, protecting organisms from further ingesting potentially harmful foods. The overall objective of these studies was to contribute to research on the physiological and toxicological roles of the AHR.

2 REVIEW OF THE LITERATURE

2.1 The aryl hydrocarbon receptor (AHR)

The AHR is an evolutionarily ancient, evidently over 600 million-year-old protein (Hahn and Karchner 2011). It is a ligand-activated transcription factor (Burbach *et al.* 1992, Ema *et al.* 1992) and thus a regulator of gene expression, which it can either promote or repress. The AHR was first identified in Alan Poland's laboratory in the 1970s (Poland *et al.* 1976). Even prior to that, its most toxic activator, TCDD, was a recognised environmental contaminant, and known to cause chloracne in humans in occupational settings (Gasiewicz and Henry 2011). However, the molecular basis or the mode of action for this toxic effect was unknown. The first hints about the existence of the AHR came through studies related to the xenobiotic metabolising enzyme CYP1A1, then called aryl hydrocarbon hydroxylase (AHH). It was known that aromatic hydrocarbons (chapter 2.2.1) induced the expression of AHH, and that while some mouse strains were very sensitive to this effect, others appeared resistant (Nebert and Gelboin 1969, Nebert and Bausserman 1970). Later, it was uncovered that TCDD was a much more potent inducer of AHH than the compounds previously studied, and that the differences between strains were due to differing structures of the receptor that mediates the effects of these compounds, the AHR.

The AHR is renowned for mediating the toxic effects of numerous xenobiotics, but it has lately also been recognised as an indispensable regulator of normal physiology and health, particularly during development (Benedict *et al.* 2000, Fernandez-Salguero *et al.* 1995, Fujii-Kuriyama and Kawajiri 2010, Harrill *et al.* 2013, Tijet *et al.* 2006). Furthermore, completely new areas regarding AHR functioning are still being discovered. Although our understanding of the functions of this receptor has increased substantially since the 1970s, surprisingly many of the questions posed so far remain incompletely answered. Furthermore, as information regarding the functions and mechanisms of the AHR has increased, the picture has become increasingly complex. Our knowledge of the molecular pathways and their interactions continues to be limited, and the list of genes that the AHR regulates is also still expanding as research progresses.

An additional complication in achieving a comprehensive understanding of the functions of this protein is that the effects of the AHR vary greatly among, for instance, species, strains, organs and developmental stages. Furthermore, the numerous chemically diverse AHR-modulating compounds induce drastically differing effects, even among the different tissues and cell types within an organism. One of the difficulties in understanding the underlying mechanisms is that the exact three-dimensional structure of the AHR remains to be established. In particular, detailed information on the spatial structure of the ligand-binding domain (LBD), as

well as the differences in this structure among different species and strains, will probably further help us to understand some of the molecular mechanisms of the AHR.

The AHR is present in most cell types across vertebrates, including humans (Hahn and Karchner 2011). While, for instance, humans, rats and mice have one AHR gene, some other mammals have two, and other vertebrates may have up to five (Gasiewicz and Henry 2011, Hahn *et al.* 2017). Moreover, AHR homologues are also widely present in invertebrates such as the nematode *C. elegans*, the fruit fly *D. melanogaster*, clams and sea urchins (Hahn *et al.* 2017). Invertebrates appear to possess single AHR genes, but, interestingly, the corresponding protein receptors do not recognise TCDD or other dioxins (Gasiewicz and Henry 2011), and as such appear to have purely physiological functions in them. Although non-mammalian AHRs are an interesting area, only vertebrate and more specifically mammalian AHRs and their functions are further discussed here due to the scope of this study. For information on AHR functions in fish and birds, Simonich and Tanguay (2011) have written an excellent review, and Powell-Coffman and Qin (2011) likewise on AHR functions in invertebrates.

2.1.1 AHR signalling and its regulation

Although at present incompetently understood at the molecular level, AHR signalling appears to involve two distinct types of pathways: canonical and non-canonical. The canonical pathway is dependent on the AHR directly binding to DNA at AHR-responsive elements [AHREs; also called dioxin-responsive elements (DREs) or xenobiotic-responsive elements (XREs)], and subsequent gene transcription. Non-canonical pathways, of which there seem to be several, involve the AHR interacting with other transcription factors or pathways within cells. It is likely that differences in mechanistic steps related to the signalling pathways and their regulation are important for the diverse outcomes of AHR modulation (Bonati *et al.* 2017, Gasiewicz and Henry 2011).

Canonical AHR-signalling pathway. The canonical, also known as the classical AHR pathway is the major AHR-signalling pathway and its best understood molecular mechanism. It was first described in connection with the induction of the xenobiotic metabolising enzyme CYP1A1 (Figure 1), but it is believed to represent a more general pattern (Q. Ma 2011). The canonical pathway encompasses activation of the AHR by ligand binding in the cytosol, and the activated AHR then moving into the nucleus. There, it pairs with a structurally related protein, aryl hydrocarbon receptor nuclear translocator (ARNT), and the AHR–ARNT complex subsequently attaches to AHREs in DNA. This leads to the recruitment of coactivators, rearrangement of chromatin and modulation of gene expression, either its activation

or repression. Although the canonical signalling pathway is generally quite well understood, many details, especially about its regulation, are still lacking.

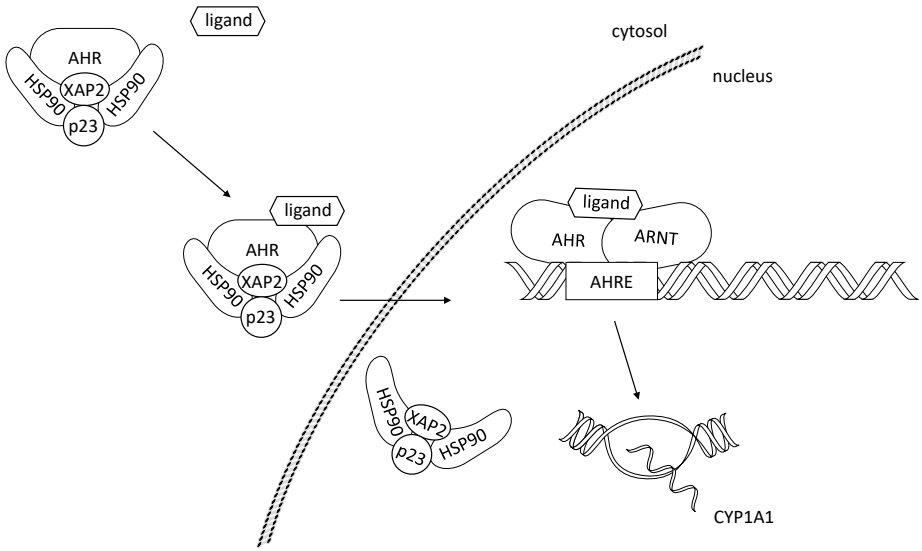


Figure 1. The canonical pathway of AHR signalling for the induction of the CYP1A1 xenobiotic metabolising enzyme (Q. Ma 2011). In its inactive state, the AHR is located in the cytosol in association with the chaperone proteins HSP90, XAP2 and p23. Binding of a ligand to the PASB domain elicits a transformation in the AHR structure, exposing a nuclear localisation sequence (NLS). The NLS is a dimerization interface for ARNT, and its exposure causes the AHR to translocate into the nucleus. There, it dimerizes with ARNT and sheds the cytosolic protein partners, transforming into a high-affinity DNA-binding form. The AHR–ARNT dimer then binds to the DNA at specific recognition sites called AHREs in the promoter region of the *Cyp1a1* gene, eventually leading to induced transcription of CYP1A1 mRNA.

Non-canonical AHR-signalling pathways. In addition to the canonical pathway, the AHR appears to act through several non-canonical pathways, which do not involve AHRE binding (Dere *et al.* 2011, Kinehara *et al.* 2008, Lo and Matthews 2012). Instead, they consist of the ligand-activated AHR interacting with other molecules within cells (Denison and Faber 2017, Guyot *et al.* 2013).

Several non-canonical pathways encompass crosstalk of the AHR with nuclear receptors and other transcription factors, such as the oestrogen receptor (ER) and nuclear factor kappa B (NF-κB), which is involved in the regulation of inflammatory pathways (Guyot *et al.* 2013, Ohtake *et al.* 2003, Patel *et al.* 2009, Vogel *et al.* 2007). An important example of such non-canonical signalling encompasses the AHR acting as a ligand-dependent E3 ubiquitin ligase (Ohtake and Kato 2011). Via this pathway,

the AHR regulates the degradation of proteins, such as the ER and β-catenin, through the ubiquitin-proteasome system. Along with other steroid hormone receptors, the ER is important, for instance, in sexual maturation, gestation, and development of hormone-related cancers. β-Catenin is vital in the canonical Wnt signal transduction cascade, which, in turn, is significant in physiology, particularly embryonic development and the pathogenesis of several diseases, including cancers (Clevers and Nusse 2012).

Furthermore, instead of heterodimerising with ARNT, the ligand-activated AHR has also been shown to pair with other nuclear proteins, such as the NF-κB-associated RelB (Denison and Faber 2017, Guyot *et al.* 2013, Vogel *et al.* 2007) and Krüppel-like factor 6 (KLF6), which is a tumour suppressor (Wilson *et al.* 2013, Wright *et al.* 2017). These distinct dimers bind to DNA sequences separate from AHREs, and regulate the expression of unique gene patterns, subsequently leading to the non-canonical regulation of gene transcription.

Non-canonical pathways also include highly complex and variable nongenomic routes, whereby ligand-activated AHR signalling is further controlled by phosphorylation via protein kinases (Guyot *et al.* 2013, Matsumura 2011). These pathways are rapid, even taking place within minutes of exposure to an AHR activator. Examples of molecules involved in these pathways include Ca^{2+}, c-src and other tyrosine kinases, cAMP-dependent protein kinases (PKAs), ERK kinase, arachidonic acid and cytosolic phospholipase A2, reviewed extensively by Matsumara (2011).

Regulation of AHR signalling. While the AHR is present in practically all mammalian tissues, its expression levels greatly differ among cell types, tissues and developmental stages (Harper *et al.* 2006). There must thus be fine-tuned mechanisms that regulate the expression of the AHR, which may also involve differential regulation of the AHR gene (Gasiewicz and Henry 2011). Sequencing of the mouse, rat and human AHR genes in the early 1990s already suggested multiple possible transcription factor binding sites (Eguchi *et al.* 1994, Garrison and Denison 2000, Gasiewicz and Henry 2011, Mimura *et al.* 1994), indicating that the *Ahr* could be up- and down-regulated in several different ways. Although a lot still remains to be elucidated about the mechanisms regulating AHR expression, many agents have been found to be involved. In addition to endogenous and exogenous compounds, these include physiological molecules such as hormones, growth factors and cytokines, and different conditions within the organism, for instance hypoxia, cell density, cell differentiation, neoplastic transformation, the developmental stage and age (Harper *et al.* 2006).

Several distinct, active mechanisms have been shown to control the negative feedback of AHR function, leading to its down-regulation. Many of these mechanisms entail autoregulation, *i.e.* involve regulatory genes whose expression is induced by ligand-activated AHR signalling, eventually down-regulating it. One of these is

Cyp1a1, further discussed in chapter 2.3.2 under *Xenobiotic metabolising enzyme induction*. Another example of such gene products is TCDD-inducible poly (ADP-ribose) polymerase (TiPARP). This belongs to the family of PARP enzymes, which have important cellular roles in, for instance, DNA repair, cell proliferation and cell death (Q. Ma *et al.* 2001, Morales *et al.* 2014). TiPARP acts as a transcriptional repressor of the AHR and directly regulates AHR activity via ADP-ribosylation (MacPherson *et al.* 2013, Matthews 2017). Another down-regulator of the AHR is aryl hydrocarbon receptor repressor (AHRR), which is a bHLH-PAS family protein like the AHR, and also directly up-regulated by ligand-activated AHR (Baba *et al.* 2001, Gasiewicz and Henry 2011, Mimura *et al.* 1999). However, the mechanism by which it restricts AHR activity is poorly understood at present (Evans *et al.* 2008, Fujii-Kuriyama and Kawajiri 2011). Furthermore, proteolytic degradation of the AHR occurs through ubiquitination, resulting in the disintegration of the AHR–ARNT complex and shortening of the half-life of activated AHR (Q. Ma and Baldwin 2000).

2.1.2 Structure of the AHR

Despite functionally resembling ligand-activated nuclear receptors, such as the ER, structurally the AHR belongs to another subfamily of transcription factors called the basic helix-loop-helix/PER-ARNT-SIM proteins (bHLH-PAS). The bHLH-PAS family includes proteins that are involved in many physiological and developmental processes. These are particularly related to the sensing of and response to environmental and cellular signals, such as xenobiotics, hypoxia, circadian rhythms, appetite control, neurogenesis and synapse formation (Furness *et al.* 2007, Gasiewicz and Henry 2011, Gu *et al.* 2000). Several members of the family are also linked to human diseases, most consistently to cancer, but also, for instance, to metabolic syndromes and psychiatric conditions (Bersten *et al.* 2013, Wu and Rastinejad 2017). In addition to the AHR, so far only one other member of the family (hypoxia-inducible factor 1α) has been shown to bind and be activated by small-molecule ligands (Wu and Rastinejad 2017). However, a considerable amount remains to be learned about the bHLH-PAS family, and there is good reason to hypothesize that other members also bind ligands, which would make the family a new and interesting target for pharmacological research.

The mouse, rat and human AHRs contain ~800–850 amino acids. Like other bHLH-PAS proteins, the structure of the AHR (Figure 2) includes an amino-terminal with a bHLH domain and two PAS domains, A and B, all of which are evolutionarily relatively conserved among species (Bonati *et al.* 2017, Gasiewicz and Henry 2011, Gu *et al.* 2000). The bHLH domain participates in dimerization with ARNT in the nucleus, which is essential for the ability of the ligand-activated AHR to bind to AHREs in DNA, and to induce transactivation (Hoffman *et al.* 1991, H. Ko *et al.* 1996, Reyes *et al.* 1992). In addition, bHLH is responsible for DNA binding. The two PAS

domains form a generic three-dimensional fold capable of small-molecule binding and protein–protein interactions. These, especially PASB, are responsible for ligand binding (Pandini *et al.* 2007) and interactions with cellular chaperones, such as the 90-kDa heat shock protein (Hsp90), which is crucial for conformational stability of the cytosolic AHR (Murray and Perdew 2011). In addition, the PAS domains participate with bHLH in the dimerization of the AHR with ARNT, and DNA binding once the AHR–ARNT complex has been formed.

The transactivation domain at the carboxyl-terminal is significant in target gene regulation and therefore signal transduction, and is generally quite poorly conserved and variable among species (Dolwick *et al.* 1993).

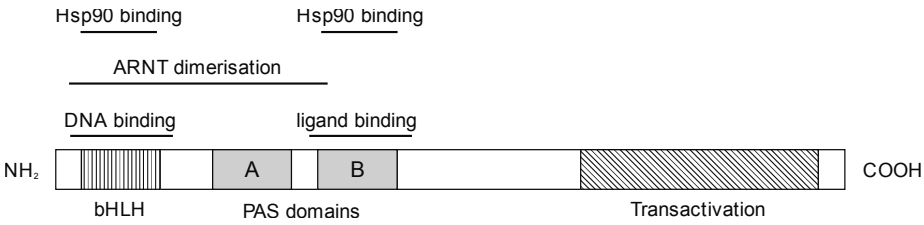


Figure 2. Major domain structures of the AHR, and approximate locations of binding regions. Modified from (Q. Ma 2011) according to (Bonati *et al.* 2017).

Even small changes in the structure of the AHR can give rise to differing ligand binding affinities, which may impact on subsequent AHR functioning. Within the LBD, the amino acid residues of the ligand-binding pocket can be responsible for the differences in binding. For instance, a sensitive and a resistant mouse strain (C57BL/6 and DBA/2J, respectively) with an approximately ten-fold difference in TCDD-binding affinity were reported to differ from each other by merely a single mutation within the LBD, resulting in one amino acid (Ala) turning into another (Val) in the resistant strain (Ema *et al.* 1994, Okey *et al.* 1989). Furthermore, as in the resistant DBA/2J mice, in the human AHR the corresponding amino acid is also Val, which is consistent with the view that humans are relatively resistant to the effects of TCDD (further discussed in chapter 2.3.1.2). Moreover, in the human LBD, a mutation turning this Val into Asp completely abolishes its ligand-binding activity (Ema *et al.* 1994).

Variability in the transactivation domain at the C-terminal of the AHR may also help explain some of the differences seen between and within species. In rats, a 1000-fold difference exists between two different strains in the TCDD doses required to elicit certain responses (Pohjanvirta *et al.* 2011). This is due to a single point mutation at the beginning of intron 10 of a TCDD-resistant Han-Wistar (*Kuopio*; H/W) strain, and consequent disruption of the normal exon–intron junction, leading to altered

mRNA splicing producing two unique AHR variants, a total loss of about 40 amino acids at the protein level, and altered transactivation domain structures (Pohjanvirta *et al.* 1998).

The only AHR domain whose complete structure has been experimentally determined thus far is PAS-A (Wu *et al.* 2013). The 3D crystal or NMR structures of the other functional domains are lacking, including the structure of the AHR LBD, PAS-B. However, *in silico* models have been developed based on existing structural information from homologous systems and sequence alignment (Bonati *et al.* 2017, DeGroot *et al.* 2011). They allow predictions of the structures and properties of the functional domains, which can then be experimentally tested, for instance, by mutagenesis and functional analysis.

2.2 AHR modulators

Unlike many other ligand-activated transcription factors, the AHR is notably promiscuous and binds a large variety of ligands with diverse structures, and of both exogenous and endogenous origin. This promiscuity is considered to be at least partly due to differences in binding to the LBD (DeGroot *et al.* 2011). Furthermore, it is particularly interesting because the effects of AHR modulation are ligand specific (Denison and Faber 2017) and of great variety, ranging from beneficial to toxic.

AHR modulators can either be full or partial agonists that activate, or antagonists that inactivate the receptor. In addition to these ligands, which exert their effects through binding with the AHR, there are also compounds that activate the AHR signalling pathway without directly binding to the receptor (Ledirac *et al.* 1997, Lesca *et al.* 1995). This can ensue, for instance, through the inhibition of AHR-mediated degradation of endogenous AHR agonists, leading to sustained secondary AHR activation (Wincent *et al.* 2012).

2.2.1 Xenobiotic modulators

AHR-modulating xenobiotic compounds are ubiquitous in our environment, and we are constantly exposed to them. Many are toxic to various degrees, while others are harmless or even beneficial to health. Xenobiotic AHR modulators belong to vastly different chemical groups and have diverse structures. They include, for instance, environmental contaminants, dietary compounds and manufactured products, such as chemicals and pharmaceuticals.

The best known and most studied role of the AHR so far is its indispensable involvement in the mechanisms of toxicity of a group of environmental contaminants encompassing halogenated organic compounds. Of these, dioxins and structurally related compounds, such as polycyclic aromatic hydrocarbons (PAHs), are

particularly important due to their toxicity and widespread distribution in the environment.

Dioxins and dioxin-like activators. Dioxins and dioxin-like compounds are environmental contaminants. They are subgroups of the highly toxic persistent organic pollutants (POPs), whose manufacture and use has been restricted by the international community since the beginning of 2000s, with the aim of banning them worldwide. Dioxins and dioxin-like compounds are distributed all around the world, and are predominantly found in soils and sediments. Furthermore, they can enter the food chain, where they biomagnify.

The subgroups of dioxins and dioxin-like AHR-activating ligands can be further divided into polychlorinated dibenzodioxins (PCDDs; dioxins), polychlorinated dibenzofurans (PCDFs; furans) and polychlorinated biphenyls (PCBs), depicted in Figure 3. Each of these three groups consists of many compounds that have the same general structure but include a different number of chlorine substituents at different positions. Compounds that differ in both the total number and position of the chlorine substituents, representing all possible PCDD, PCDF or PCB compounds, are called congeners. The toxicity of different congeners greatly differs depending on their chemical structure, ultimately resulting in differences in AHR-binding affinities and pharmacokinetics (Safe 1990).

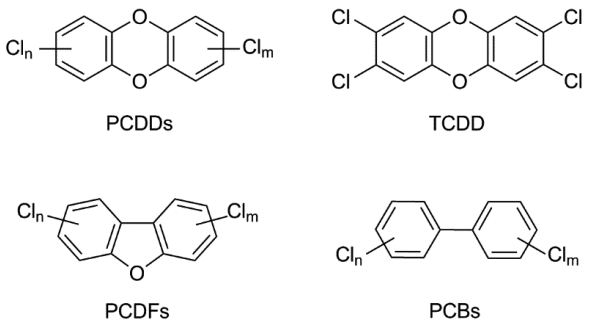


Figure 3. Chemical structures of polychlorinated dibenzodioxins (PCDDs), the most potent dioxin congener 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs).

PCDDs include 75 congeners, of which 7 are particularly toxic, and PCDFs include 135 congeners, 10 of which have dioxin-like properties. Both general structures may contain up to eight chlorine substituents. The congeners that result in characteristic dioxin-like toxicity have four to eight chlorine substituents, four of which are positioned in the lateral positions 2, 3, 7 and 8 (Safe 1990). PCBs exist as 209

congeners, a dozen of which are AHR agonists and considered dioxin-like due to their toxicity. This results from their structure, with a minimum of four chlorine substituents that are positioned co-planarly, rendering these compounds as rigid structures that are similar to PCDDs and PCDFs. Collectively, the 29 particularly toxic PCDD, PCDF and PCB congeners are often referred to as "dioxins".

The most toxic congener is TCDD (Figure 3), which is considered the most toxic synthetic chemical ever produced, based on its lethality in the most sensitive species, the guinea pig [LD50 ~1 μg/kg, (Schwetz *et al.* 1973)]. TCDD is a PCDD with four chlorine atoms in the above-mentioned positions. It has, as such, been widely employed in research as the classical compound for activation of the AHR, and a model compound for dioxin toxicity (further described in chapter 2.3.1.1).

PCBs were previously widely manufactured and used in industry, mostly as mixtures, due to their non-flammability, chemical stability, high boiling point and electrical insulating properties. PCDDs and PCDFs, however, have never been manufactured deliberately, apart from research use. Nevertheless, they are easily formed unintentionally as by-products of industrial processes, including chlorine bleaching of paper pulp, smelting and the manufacture of chlorinated chemicals, and also due to incomplete combustion, for instance from uncontrolled waste incineration. In addition, PCDDs and PCDFs occur as impurities among other chlorinated chemicals, such as PCBs and chlorophenols.

All of these compounds are chemically highly stable and resistant to biodegradation, which leads to their persistence in the environment. In addition, they are highly hydrophobic and accumulate in the food chain, and eventually also in humans (Kiviranta, *et al.* 2002, Travis and Hattemer-Frey 1991). Their half-lives in adult humans ranges up to 13 years (Milbrath *et al.* 2009). Dietary sources of dioxins are mainly fatty animal products, typically dairy products, eggs, meat and fish (J. Tuomisto *et al.* 2011).

Polycyclic aromatic hydrocarbons (PAHs). PAH compounds naturally occur in coal, crude oil and gasoline. They are also produced in the burning of, for instance, coal, oil, gas, wood, garbage and tobacco. In addition, cooking of food, particularly meat, at high temperatures produces PAHs. Like dioxins, PAHs are AHR ligands and several are commonly employed as AHR activators in research. A representative example is benzo-*a*-pyrene (BaP; Figure 4), a carcinogen that humans are typically exposed to from tobacco smoke and residential wood burning.

While not a PAH compound due to the two oxygen molecules in its structure, β-naphthoflavone (BNF; Figure 4) is another potent AHR agonist commonly used in research. It is a polyaromatic, PAH-type inducer of the AHR.

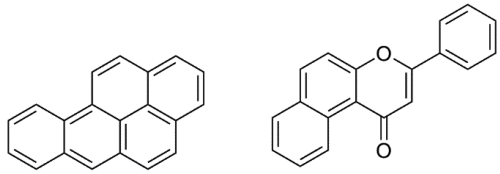


Figure 4. The chemical structures of the AHR agonists benzo-*a*-pyrene (BaP) and β-naphthoflavone (BNF), which are commonly used in research.

Dietary AHR modulators. In addition to the AHR-activating chemical contaminants in food, such as dioxins and PAHs, many naturally occurring AHR-modulating compounds are also present in the human diet. They are commonly found in vegetables, fruits, berries, tea and herbal food extracts such as ginseng, gingko balboa and liquorice (Safe *et al.* 2011). Dietary AHR modulators can exhibit either AHR-activating or -inactivating properties, and several are partial agonists. In addition, many appear to be SAHRMs (further described below), exhibiting differing activities depending on, for instance, the exposure time, cell type and species (Safe *et al.* 2011, Van der Heiden *et al.* 2009).

Among the major sources of AHR modulators in the human diet are polyphenolics (Gasiewicz and Henry 2011). Flavonoids are a large family of polyphenolic compounds that are widely present in vegetables and generally consumed in significant amounts (González *et al.* 2011). They have anti-inflammatory activity, and many are antioxidants. Examples of AHR-modulating flavonoids include debenzoylmethane, a constituent of liquorice, and several compounds found in green and black tea (Safe *et al.* 2011). Other types of AHR-modulating polyphenols, structurally loosely related to flavonoids, include curcumin, an anticancer compound found in turmeric spice (Aggarwal *et al.* 2003), and resveratrol, found in many fruits and berries, and in particular abundance in red grapes (González *et al.* 2011, Safe *et al.* 2011). Resveratrol has been extensively studied due to its wide array of protective effects, including strong antioxidant, anti-inflammatory, and anticancer properties (M. Jang *et al.* 1997).

Several AHR-binding dietary compounds have been associated with health benefits (Safe *et al.* 2011). For instance, indole-3-carbinol (I3C), present in cruciferous vegetables such as cabbage and broccoli, and its several AHR-modulating metabolites arising *in vivo*, have been linked with the antitumorigenic activity of these vegetables (Bjeldanes *et al.* 1991, Chen *et al.* 1996, Chen *et al.* 1998). Also resveratrol has been shown to inhibit PAH-initiated tumorigenicity *in vivo* (M. Jang *et al.* 1997). Furthermore, it appears to prevent TCDD-induced developmental toxicity in mice, including cleft palate, renal pelvic dilation, and ureteric dilatation and tortuosity (J. Y. Jang *et al.* 2008, Safe *et al.* 2011), thus acting as an AHR

antagonist. On the other hand, in the mouse model for studying multiple sclerosis [MS; experimental autoimmune encephalomyelitis (EAE) model], resveratrol exhibits AHR-inducing activity, showing a protective effect similar to that of TCDD, and thus also agonistic activity (Quintana *et al.* 2008, N. P. Singh *et al.* 2007).

Dietary AHR modulators characteristically have numerous cellular targets in the body, the AHR being only one of them. Furthermore, most of these compounds have only low to moderate AHR-binding affinities and CYP1A1-inducing potencies, typically >100 to 1000 times lower than that of TCDD (Safe *et al.* 2011). They are therefore unlikely to be highly important as physiological AHR ligands individually (Gasiewicz and Henry 2011). However, considering that small amounts of many of these compounds are constantly present in our diet, it is conceivable that they could have additive AHR-mediated effects.

AHR antagonists and partial agonists. In addition to compounds that activate the AHR, there are compounds that bind to the AHR without causing effects, and are able to block or reduce the effects of AHR agonists, called AHR antagonists (Gasiewicz and Henry 2011, Keys *et al.* 1986). Most AHR antagonists compete with agonists for the same binding site, but several other mechanisms exist (Gasiewicz and Henry 2011, Henry *et al.* 1999, Nishiumi *et al.* 2007, Palermo *et al.* 2005). Examples of AHR antagonists include CH-223191 and GNF351, depicted in Figure 5.

CH-223191 is a potent, ligand-selective inhibitor of AHR activation in various species (S. -. Kim *et al.* 2006, Zhao *et al.* 2010). It has been shown *in vitro* to block the binding of TCDD to the AHR, to inhibit its nuclear translocation and binding to AHREs, and to inhibit xenobiotic metabolising enzyme induction by TCDD (S. -. Kim *et al.* 2006). Furthermore, *in vivo*, in addition to significantly reducing the TCDD-induced induction of xenobiotic metabolising enzymes, CH-223191 was shown by Kim and colleagues to prevent liver toxicity and wasting syndrome in mice. Moreover, CH-223191 appears to be a competitive, pure AHR antagonist with no detectable AHR-agonist activity. However, it has been shown to be ligand selective, fully antagonising only halogenated aromatic hydrocarbons, such as TCDD and PCBs, but not non-halogenated AHR agonists, such as PAHs, BNF or flavonoids (Zhao *et al.* 2010).

GNF351 has also been reported to be a competitive, complete AHR antagonist *in vitro* (Smith *et al.* 2011). It was demonstrated to be a potent, high-affinity ligand of the AHR, and to also be able to antagonise AHR activity related to both canonical and cytokine-mediated non-canonical signalling. Furthermore, GNF351 appears to be a non-selective antagonist. However, following oral exposure *in vivo*, the antagonising effect of GNF351 was limited to the gastrointestinal tract in mice (Fang *et al.* 2014). This was reported to be due to poor absorption and extensive metabolism.

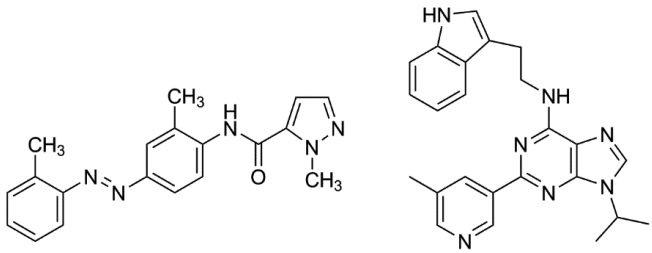


Figure 5. Chemical structures of the AHR antagonists CH-223191 and GNF351.

Not all AHR-modulating compounds are either agonists or antagonists; partial AHR agonists can act as both, depending on their concentration. In fact, most, if not all, of the early compounds employed as AHR antagonists, such as α-naphthoflavone, have later been discovered to be partial AHR agonists (Safe *et al.* 2011). The maximum efficacy of partial agonists is less than that of full agonists, and in the presence of a full agonist, partial agonists typically block or reduce the agonist's effect by competitive binding. Partial AHR agonists are relatively common, for instance, among AHR modulators in the diet.

Moreover, AHR-modulating compounds exist that can act as antagonists in some species and as agonists in others. This is most likely, at least in part, due to structural differences in AHR orthologues among species (Aarts *et al.* 1995, Gasiewicz and Henry 2011, Henry and Gasiewicz 2008). Furthermore, even within a single species, these compounds can have either or both agonist/antagonist activity, depending on the tissue. Such compounds are called selective AHR modulators (SAHRMs).

Selective AHR modulators (SAHRMs). The term SAHRM has not been unambiguously defined, but it is generally used to describe compounds that exhibit tissue- or species-specific AHR activation or inactivation, or only induce some of the typical responses of AHR activation, but not others. The term is sometimes also used more specifically to describe compounds that exhibit AHR-mediated effects, such as anti-inflammatory properties, solely through non-canonical signalling pathways (Murray *et al.* 2010, Murray *et al.* 2011). SAHRMs may be naturally occurring compounds or engineered and optimised with the aim of inducing only favourable responses of AHR modulation.

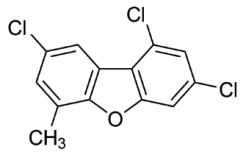
Considering the increasing evidence for involvement of the AHR in physiological functions and disease aetiology, including both pro- and antitumour pathways, it appears a highly interesting target for novel therapies employing SAHRMs. The rationale for this is based on the notion that some of the biological impacts of even TCDD are such that they would be beneficial in the treatment of certain diseases if they could be separated from the toxicity. These impacts are particularly related to

immunomodulation and cancer (Quintana *et al.* 2010, Vorderstrasse and Lawrence 2006, Xu *et al.* 2015, Zhu *et al.* 2014). Therefore, in addition to cancer (Díaz-Díaz *et al.* 2016, Jin, Lee, Pfent *et al.* 2014), appropriate activation of the AHR could lead to novel therapeutics for the treatment of, for instance, MS (Quintana *et al.* 2010, N. P. Singh *et al.* 2007), inflammatory skin diseases (Di Meglio *et al.* 2014, Haas *et al.* 2016, Van Den Bogaard *et al.* 2013), Crohn's disease (Benson and Shepherd 2011) and inflammatory bowel disease (Arsenescu *et al.* 2011, Furumatsu *et al.* 2011). In addition, SAHRMs can be useful tools in the quest of further elucidating the molecular mechanisms at play in the biological and toxicological roles of the AHR.

The exact mechanisms of SAHRMs in tissue specificity are yet to be elucidated, but it is conceivable that they could resemble those of selective modulators of nuclear receptors, which exhibit a similar function as transcription factors to the AHR. For hormone-binding nuclear receptors, such as the ER, distinct mechanisms for selectivity have been established. These are due to multiple factors, including a) differential metabolism of ligands among different tissues, b) multiple receptor forms, splice variants, and differential patterns of receptor dimerization, in part depending on the ligand, and c) differences in the conformational changes of the receptor, and in the subsequent interactions with nuclear cofactors, depending on the ligand and/or tissue (Katzenellenbogen *et al.* 1996, Safe *et al.* 2013).

A representative example of SAHRMs is one of the early engineered compounds, 6-methyl-1,3,8-trichlorodibenzofuran (MCDF; Figure 6). MCDF binds to the AHR, but they form a complex that is transcriptionally inactive. Furthermore, MCDF has been shown to inhibit the induction of the xenobiotic metabolising enzyme CYP1A1 by TCDD both *in vitro* and *in vivo*, and also to inhibit several classical TCDD-induced toxic responses, such as immunotoxicity and developmental toxicity (Astroff *et al.* 1988, Bannister *et al.* 1989, M. Harris *et al.* 1989, Safe *et al.* 2011). Thus, MCDF clearly exhibits AHR-antagonistic activity.

However, MCDF does not inhibit TCDD-induced, AHR-mediated antioestrogenic activity (described further under *Carcinogenicity* in chapter 2.3.1.1). In contrast, it acts as an AHR agonist in multiple oestrogen-responsive tissues in several species, showing antioestrogenic activity much like TCDD (Astroff and Safe 1991, Safe *et al.* 2011, Zachrewski *et al.* 1992). This is interesting, because antioestrogenic compounds can be used in the treatment of hormone-dependent tumours, which are characteristic of, for instance, ER-positive breast cancer (Lerner and Jordan 1990). Correspondingly, MCDF has been shown to be a potent inhibitor of carcinogen-induced mammary tumour growth in rats (McDougal *et al.* 1997). It has also been reported to inhibit prostate tumour metastasis in a mouse model (Fritz *et al.* 2009). In addition to MCDF, other SAHRMs have also been shown to inhibit the growth of hormone-dependent cancers, apparently without AHR-related toxicity (McDougal *et al.* 2001, Safe *et al.* 2011, S. Zhang *et al.* 2009).



MCDF

Figure 6. Chemical structure of SAHRM 6-methyl-1,3,8-trichlorodibenzofuran (MCDF).

Pharmaceuticals. Many drug targets are ligand-activated proteins, for instance G protein-coupled receptors or nuclear receptors. However, for a long time, the AHR was not seen as a potential drug target due to the view that AHR activation would inevitably lead to toxic consequences. Subsequently, this view has been overturned, and the AHR is presently seen as a highly potential drug target. Furthermore, there are in fact several existing drugs on the market that, while primarily modulating other pathways, have also been found to affect AHR activity (Table 1). This is not surprising given the present literature illustrating the varied functions of the AHR in physiology within organisms.

Table 1. Examples of AHR-active drug compounds on the market

Compound	Drug class	Indication(s)	References
Flutamide	Antiandrogens	Prostate cancer	(Koch *et al.* 2015)
Itraconazole, Ketoconazole	Antifungal drugs (azole derivatives)	Fungal infections	(Korashy, Shayeganpour *et al.* 2007, Korashy, Brocks *et al.* 2007)
Leflunomide	Disease-modifying antirheumatoid drugs	Rheumatoid arthritis, psoriatic arthritis	(O'Donnell *et al.* 2010)
Mexiletine	Antiarrhythmic drugs (non-selective sodium channel blockers)	Heart arrhythmias	(Jin *et al.* 2012)
Nimodipine	Calcium channel blockers	Prevention of cerebral vasospasm and ischemia following subarachnoid bleeding	(Jin *et al.* 2012)
Omeprazole	Proton pump inhibitors	Gastroesophageal reflux disease, peptic ulcer disease	(Dzeletovic *et al.* 1997, Quattrochi and Tukey 1993)
Sulindac, Salicylamide	Nonsteroidal anti-inflammatory drugs	Pain and inflammation	(Ciolino *et al.* 2006, MacDonald *et al.* 2004)
Transilast	Antiallergic drugs	Asthma, allergic rhinitis, atopic dermatitis	(Prud'Homme *et al.* 2010)

Many of the AHR-modulating drug compounds on the market are SAHRMs (Jin *et al.* 2012). Several are anti-inflammatory compounds, but others have also been reported. An interesting example is 4-hydroxytamoxifen, one of the major active anticancer metabolites of the classical breast cancer drug tamoxifen, which is a selective ERα-antagonist. 4-Hydroxytamoxifen is currently under development as a product, and therefore not yet on the market as a distinct drug compound, but it has been extensively studied in conjunction with tamoxifen. In addition to exhibiting anti-cancer properties, 4-hydroxytamoxifen has been shown to be an AHR ligand that modulates its transcriptional activity (DuSell *et al.* 2010). Therefore, through a metabolite, the mechanism of action of tamoxifen also appears to involve the modulation of the AHR (Gasiewicz and Henry 2011).

Furthermore, numerous compounds are being studied as potential AHR-modulating drug compounds, primarily for the treatment of different cancers and inflammatory diseases (Ehrlich and Kerkvliet 2017, Safe *et al.* 2013, Safe *et al.* 2017). Among these compounds is laquinimod, for which C1, one of the novel SAHRMs studied in this thesis, is an *N*-hydrogen metabolite. Laquinimod has been studied in phase II/III clinical trials for efficacy and safety in the treatment of MS (Polman *et al.* 2005, Thöne and Linker 2016) and Crohn's disease (D'Haens *et al.* 2015), and is currently in phase II studies for the treatment of Huntington's disease (Garcia-Miralles *et al.* 2016). The mechanism of action of laquinimod is not yet fully elucidated, but it has been recognised as an immunomodulatory compound (Varrin-Doyer *et al.* 2014), and the mode of action has been shown to be AHR dependent in the mouse EAE MS model (Berg *et al.* 2016, European Medicines Agency 2014, Kaye *et al.* 2016)

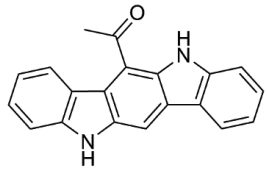
2.2.2 Endogenous modulators

In addition to being promiscuous in ligand binding, another aspect that distinguishes the AHR from many other ligand-activated transcription factors is that, despite research efforts, no single endogenous substance has thus far stood out as its primary physiological activator. However, due to the functions of the AHR in normal physiology (chapter 2.3.2), it seems apparent that there must be one or several major endogenous activators of the AHR.

There is one particularly interesting candidate group of compounds: metabolites and/or photo-oxidation products of tryptophan. Tryptophan is an essential amino acid that is present in most dietary proteins. It is the precursor for several important compounds in the body, such as serotonin, melatonin and niacin. Further derivatives include other indole-based compounds, many of which are AHR ligands (Heath-Pagliuso *et al.* 1998, Helferich and Denison 1991, A. Rannug *et al.* 1987). Of these, 6-formylindolo(3,2-b)carbazole (FICZ; Figure 7) appears particularly interesting due to

its potency and exceptionally high affinity for the AHR, which are comparable to those of TCDD (A. Rannug *et al.* 1987, Wei *et al.* 1998, Wincent *et al.* 2009). Regardless of these characteristics, FICZ is not toxic. In contrast, FICZ has been shown to ameliorate asthma symptoms and inflammatory skin conditions, such as psoriasis, in mouse models (Di Meglio *et al.* 2014, Jeong *et al.* 2012).

Another two noteworthy tryptophan-metabolite AHR ligands are kynurenine and kynurenic acid, which appear to be particularly important in promoting AHR-related carcinogenesis (DiNatale *et al.* 2010, Opitz *et al.* 2011). A potent, non-toxic agonist called 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), on the other hand, has anticancer properties (Henry *et al.* 2006, Song *et al.* 2002, K. Wang *et al.* 2013). ITE has also been reported to have other protective properties, including the potential amelioration of several autoimmune disorders, such as uveitis, colitis and MS (Goettel *et al.* 2016, Nugent *et al.* 2013, Quintana *et al.* 2010). Further endogenous AHR agonists and antagonists include bilirubin, biliverdin, 7-ketocholesterol, prostaglandins and arachidonic acid derivatives (Gasiewicz and Henry 2011, Safe *et al.* 2011)



FICZ

Figure 7. Chemical structure of the endogenous AHR agonist 6-formylindolo(3,2-b)carbazole (FICZ).

2.3 AHR-mediated effects

The AHR is noteworthy as a transcription factor due to the diversity of modulators it binds. Moreover, the vast and varied array of functions it has been shown to mediate in organisms, both toxicological and physiological, further increases its importance in biology. However, understanding AHR-mediated effects in full has proved challenging due to the complexity of its function on several levels.

Differences among species and strains. While AHR homologues are widespread in fauna, their functions among species vary. The expression of certain genes, such as *Cyp1a1*, is consistently modulated by the AHR across species in response to AHR modulators (Denison and Faber 2017). However, there are also various genes whose expression differs significantly among species (Flaveny *et al.*

2010, Forgacs *et al.* 2013, Kovalova *et al.* 2017, Sun *et al.* 2004). Most strikingly, a difference of over 2000-fold in TCDD lethality has been reported between the most sensitive and resistant species (guinea pig and hamster, respectively), represented as LD50 values, which denote the doses estimated to kill 50% of animals in an acute toxicity study. For guinea pigs, the LD50 value is 0.6–2 μg/kg (M. W. Harris *et al.* 1973, McConnell *et al.* 1978, Schwetz *et al.* 1973), while for hamsters it is reported to be as high as ~5000 μg/kg (Henck *et al.* 1981).

Even within species, AHR-modulator effects can differ among strains. The largest within-species difference in sensitivity to TCDD has been reported in rats, in which a difference of at least a 1000-fold exists between the most sensitive and resistant strains described [LD50 values ranging from 10–20 to >9600 μg/kg in Long-Evans (*Turku/AB*; L-E) and H/W (*Kuopio*) rats, respectively (Pohjanvirta *et al.* 1993, Unkila *et al.* 1994)].

The strain and species differences in dose-dependent sensitivity to given AHR modulators appear to be largely attributable to structural differences in the AHR (Denison *et al.* 2011, Gasiewicz and Henry 2011, Pohjanvirta *et al.* 2011, Romkes *et al.* 1987). As described in chapter 2.1.2, even small changes in the structure of the protein may give rise to differences in its ligand-binding ability and transactivation, and subsequently the effects the AHR mediates. However, structural differences may not be adequate to explain the diversity of AHR-mediated molecular responses observed within organisms among, for instance, cell types or organs, sexes, age groups, or developmental stages, which further complicates the overall picture.

Differences among AHR modulators. There is vast diversity in AHR-mediated effects depending on which modulator is responsible for initiating them, as already established in chapter 2.2. Although the sensitivity to TCDD among species and strains correlates with differences in the structure of the LBD, and consequently binding affinity (Pohjanvirta *et al.* 2011), this is not the case with AHR modulators in general. For example, the endogenous tryptophan metabolite FICZ is not toxic, much unlike TCDD, even though FICZ binds to the AHR *in vitro* with even somewhat greater affinity than TCDD (K_d values of 0.07 and 0.48 nM, respectively) (A. Rannug *et al.* 1987, U. Rannug *et al.* 1995). The differences in effects following the binding of these two compounds are probably, at least in part, due to kinetics. The metabolism of FICZ is extensive and rapid *in vivo* (Bergander *et al.* 2003, Bergander *et al.* 2004), whereas TCDD has a very long half-life, consequently bringing about prolonged activation of the AHR (Abraham *et al.* 1988, Rose *et al.* 1976). It appears that kinetics is also more generally a major contributor to the effects that different compounds produce within organisms (Van den Berg *et al.* 1994).

Moreover, it appears that different AHR ligands, and consequently signalling pathways, can regulate the expression of distinct gene patterns (Denison and Faber 2017). For instance, the AHR has been shown to regulate two separate gene batteries in mice: those dependent on TCDD and those independent of it (Tijet *et al.* 2006).

This could reflect the two distinct outcomes of activation of the receptor: mediation of the toxic effects of xenobiotics (below), and the maintenance of normal physiological functions (chapter 2.3.2).

2.3.1 Toxic effects mediated by the AHR

AHR-mediated toxicity is presented here using TCDD as a model compound. This is partly due to the complexity of AHR functioning, but also because of the scope of this study. Furthermore, TCDD is the most toxic AHR activator, and has therefore been extensively used in studying AHR-mediated toxicity.

The toxic effects following TCDD exposure are almost exclusively dependent on the AHR (Fernandez-Salguero *et al.* 1996, Harrill *et al.* 2016, Mimura *et al.* 1997, Nishimura *et al.* 2005, Vorderstrasse *et al.* 2001), and practically all of the so far elucidated toxicities entail canonical signalling (Bunger *et al.* 2003, Bunger *et al.* 2008, Tijet *et al.* 2006). However, it is possible that there are yet to be established toxic effects of TCDD that involve the non-canonical pathways. Candidates are, for instance, effects that occur through epigenetic mechanisms.

As with the details of AHR signalling in general, the pathways responsible for the toxic effects of TCDD are still incompletely understood. The current consensus is that the toxicity results from inappropriately sustained and untimely activation of the AHR (Bock and Köhle 2006, Denison *et al.* 2011). Furthermore, it appears clear that it is a consequence of simultaneous dysregulation of multiple genes and signalling pathways, and as such very complex (Gasiewicz and Henry 2011). For instance, there are hundreds of genes in different tissues whose expression has been reported to be modified by TCDD, and these genes differ among species (Boutros 2011).

The differences in TCDD susceptibility among species and strains mostly appear to be due to differences in AHR structure (Ema *et al.* 1994, Korkalainen *et al.* 2000, Pohjanvirta *et al.* 1998). Differences among organs and developmental stages within species and strains, on the other hand, may reflect the TCDD susceptibility of particular tissues. Many of the affected organs, tissues and cells are those undergoing differentiation and proliferating quite rapidly, such as epithelial and mucosal tissues, skin (in humans), spermatozoa and the immune system (Merches *et al.* 2017, Mocarelli *et al.* 2011, Stockinger *et al.* 2014). This is consistent with findings of TCDD altering the cell cycle, patterns of cellular proliferation and differentiation, and cell–cell communication (Gasiewicz and Henry 2011). Furthermore, in most cases, the embryo and foetus are much more susceptible to the toxicity of TCDD than adults (Bock and Köhle 2006). Additionally, in developing organisms, inter- and intra-species variability in sensitivity to TCDD-induced lethality is much less pronounced than in adults, with a difference of only ~10-fold even between the most sensitive and resistant species (Huuskonen *et al.* 1994, Kransler *et al.* 2007).

2.3.1.1 TCDD toxicity in experimental animals

TCDD causes a multitude of adverse effects in laboratory animals, of which the rat and mouse are the most studied species. Characteristic toxic effects seen in one or both of these species include lethality, immunotoxicity, hepatotoxicity, endocrine disruption, developmental toxicity, reduced fertility and carcinogenicity (Pohjanvirta *et al.* 2011). Furthermore, TCDD has effects on epithelial and mucosal tissues, teeth and bones, cardiovascular health, and induces oxidative stress. Recent findings in rats, mice and zebrafish also imply that TCDD may induce transgenerational effects, such as reduced fertility (T. R. Baker *et al.* 2014, Bruner-Tran and Osteen 2011, Sanabria *et al.* 2016). A possible mechanism may be epigenetic modification of DNA (B. B. Baker *et al.* 2016, J. Ma *et al.* 2015, Manikkam *et al.* 2012), although further studies on the mechanism are warranted. Overall, it is important to note that most of the toxic effects of TCDD are species specific. Moreover, similarly to the toxic effects following chronic exposure, TCDD toxicity following a single exposure also emerges in a delayed fashion.

It is noteworthy that even species and strains that are very resistant to the lethality of TCDD, such as the H/W (*Kuopio*) rat and hamster, still display other AHR-dependent effects, much like sensitive species (Gasiewicz, Henry *et al.* 1986, Pohjanvirta *et al.* 1988, Pohjanvirta *et al.* 2011, Unkila *et al.* 1993). Based on the TCDD-sensitive L-E and TCDD-resistant H/W rat strain model of TCDD toxicity (Pohjanvirta *et al.* 2011), AHR-mediated effects can be classified as either type I or type II. Type I effects emerge at the same doses in both strains, while eliciting type II effects in H/W rats requires at least 100-fold higher doses than in L-E rats. Consequently, this means that type I effects are robust to structural variations in the AHR transactivation domain, and thereby represent more generic AHR-mediated impacts.

Type I effects include the induction of xenobiotic metabolising enzymes, thymus atrophy, foetolethality, a derailed vitamin A status, hypercholesterolemia, and reduced plasma thyroxine and melatonin levels (Pohjanvirta *et al.* 2011). Type II effects include lethality, wasting syndrome, liver toxicity and tumour promotion. As a general rule, type II effects occur within species and strains at higher doses of TCDD than type I effects, although exceptions exist.

Lethality and wasting syndrome. While the most resistant species and strains can tolerate TCDD doses of even several g/kg, lethality is seen in sensitive species and strains in the dose range of μg/kg. The pathogenesis of TCDD-related lethality continues to be largely unknown, but it appears predominantly due to body weight loss resulting from hypophagia, which can culminate in wasting syndrome (Kelling *et al.* 1985, Lindén *et al.* 2010, McConnell *et al.* 1978, Pohjanvirta *et al.* 2011, Schwetz *et al.* 1973).

Wasting syndrome is a condition seen in several species after TCDD exposure, particularly in the guinea pig and rat. It is characterised by a dramatic body weight loss of even more than 50%, leading to lethality, and further includes hepatotoxicity, hepatosteatosis and decreased gluconeogenesis (Lindén *et al.* 2010, Pohjanvirta and Tuomisto 1994). However, even at very high doses following a single dose, mortality is only seen 1–8 weeks post-exposure, depending on the dose and sensitivity of the species or strain (Lindén *et al.* 2010, Poland and Knutson 1982). Prior to that, body weight loss is progressive. Interestingly, among all AHR agonists, wasting syndrome has only been reported to be brought about by TCDD and related dioxins (Neal *et al.* 1979, Pohjanvirta and Tuomisto 1994).

Organ toxicity. Depending on the species, TCDD causes toxicity in several organs and tissues. Some of the major targets include the liver, thymus, testes, intestine and urinary tract (Pohjanvirta *et al.* 2011).

Thymic atrophy is the only TCDD-induced toxic effect that affects practically all mammals in a consistent and uniform manner (Gupta *et al.* 1973, M. W. Harris *et al.* 1973, Pohjanvirta and Tuomisto 1994, Poland and Knutson 1982). It mainly stems from the depletion of small immature cortical thymocytes (Vos *et al.* 1974) by a mechanism that may involve delayed maturation of T-lymphocyte precursors (Greenlee *et al.* 1985, Holladay *et al.* 1991), enhanced apoptosis (McConkey *et al.* 1988) and impaired thymic seeding by prothymocytes (Fine *et al.* 1990).

Hepatotoxic effects of TCDD include lesions in the liver (Kociba *et al.* 1978, Pohjanvirta *et al.* 1989, Vos *et al.* 1974), hepatocellular hypertrophy (Christian *et al.* 1986), hepatic steatosis (J. H. Lee *et al.* 2010), jaundice (Gupta *et al.* 1973), the accumulation of biliverdin (Niittynen *et al.* 2003) and altered vitamin A homeostasis (Hoegberg *et al.* 2003, C. K. Schmidt *et al.* 2003).

Even in adult rats, exposure to TCDD induces alterations in testis morphology and function, including testicular atrophy, reduced Leydig cell volume, a reduced number of spermatids and decreased spermatozoa number (Chahoud *et al.* 1992, El-Sabeawy *et al.* 1998, Johnson *et al.* 1992). In the stomach, intestine and urinary tract, epithelial and mucosal lesions are seen (McConnell *et al.* 1978, Poland and Knutson 1982). Furthermore, TCDD induces hypoglycaemia and alterations in blood lipids, including hypercholesterolemia and an elevation of free fatty acids (FFA) in plasma (Fletcher *et al.* 2005, Pohjanvirta *et al.* 1989, Potter *et al.* 1983, Simanainen *et al.* 2003).

Immunotoxicity. Even a single dose of TCDD weakens both cell-mediated and antibody-mediated adaptive immunity in rodents, increasing their susceptibility to infectious diseases and transplanted tumours (Kerkvliet 2011, Luebke *et al.* 2006, Pohjanvirta and Tuomisto 1994). Immunotoxicity occurs in mice in the range of low μg/kg, below doses that induce overt toxicity (Kerkvliet 2011). Interestingly, TCDD-induced immunotoxicity appears to be independent of thymus function, as thymectomised adult mice are also sensitive to dioxin-induced immune suppression,

which furthermore occurs at doses below those inducing thymic atrophy (Kerkvliet and Brauner 1987).

Endocrine disruption. Particularly in the rat, TCDD causes disturbances of the hormone system (Pohjanvirta *et al.* 2011). These effects include decreases in the circulating levels of testosterone, insulin and melatonin, and an increase in the level of adrenocorticotropic hormone. The level of corticosterone, the main glucocorticoid in rats, is also modulated (Dibartolomeis *et al.* 1987).

Furthermore, thyroid function is disturbed, manifested as a decrease in the circulating level of thyroxine (T4), which chiefly emanates from accelerated thyroxine catabolism by UGT1A6 in the liver (Nishimura *et al.* 2005). Furthermore, an increase in thyroid-stimulating hormone is induced (Potter *et al.* 1986). The reduction in serum thyroxine appears to be one of the most sensitive endocrine indicators of exposure to TCDD in adult rats, with an ED50 between 1 and 5 μg/kg in SD rats (Viluksela *et al.* 2004).

Developmental toxicity. As previously mentioned, all species and strains are almost equally sensitive to TCDD lethality in the embryonic stage (Kransler *et al.* 2007). Furthermore, developmental effects following TCDD exposure *in utero* are among the most sensitive endpoints overall, appearing at doses that are well below those that are toxic to the pregnant or lactating adult female (Abbott 2011). Effects observed in different species include foetal lethality, the inhibition of growth, effects on liver weight, neurobehavioural effects, endocrine disruption and malformations (Abbott 2011, Brouwer *et al.* 1995, Couture *et al.* 1990). However, the major developmental effects following TCDD exposure *in utero* largely depend on the species.

In mice, hydronephrosis and cleft palate are the characteristic and most sensitive effects (Abbott 2011). In rats, typical developmental toxicity likewise entails cleft palate, effects on teeth and bone, and disturbances in male sexual behaviour and the reproductive system (Huuskonen *et al.* 1994, Kattainen *et al.* 2001, Mably, Moore, Goy *et al.* 1992, Mably, Moore and Peterson 1992). In particular, the effects on teeth, such as disturbances in molar development, are among the most sensitive endpoints in developing rats, but they also occur in many other species (Viluksela *et al.* 2011).

Carcinogenicity. TCDD is a carcinogen in all experimental animal species tested, targeting, for instance, the liver, thyroid, lung, skin and oral cavity, depending on the species (Pohjanvirta *et al.* 2011, Schrenk and Chopra 2011). It is not genotoxic but also induces tumours after chronic treatment in the absence of tumour initiators (Kociba *et al.* 1978). In addition, TCDD acts as a tumour promoter (Pitot *et al.* 1980). The underlying mechanisms are at present mostly undefined, although at least three distinct ways have been recognised in which activation of the AHR can stimulate tumorigenesis: the metabolic activation of procarcinogenic compounds, modulation

of genes related to proliferation and the immune system, and regulation of apoptosis (Bersten *et al.* 2013, Schrenk and Chopra 2011).

The metabolic activation of procarcinogens applies, for instance, for PAHs such as BaP, which are bioactivated into reactive metabolites that form DNA adducts (Nebert *et al.* 2004). These, in turn, bind covalently to DNA, resulting in mutations that may eventually lead to cancer.

The AHR can also stimulate tumour promotion and/or progression through the modulation of genes that are related to proliferation and the immune system (Bersten *et al.* 2013). For example, in human gliomas, constitutive metabolism of tryptophan to kynurenine by tumour cells leads to AHR-mediated increased tumour-cell survival and motility, and the inhibition of antitumour immune responses via the suppression of the recruitment of tumour antigen recognizing immune cells (Opitz *et al.* 2011). This is associated with malignant progression and poor survival.

Furthermore, the inhibition of apoptosis appears to be an important mechanism in AHR-mediated carcinogenesis (Schrenk and Chopra 2011), and may prove a more accurate explanation for the tumour promoting activity of TCDD than the induction of cell proliferation (Pääjarvi *et al.* 2005, Stinchcombe *et al.* 1995). The mechanisms behind the AHR-mediated inhibition of apoptosis are at present poorly understood, but p53 appears to be a key regulator (Pääjarvi *et al.* 2005, Schrenk and Chopra 2011).

2.3.1.2 Dioxin toxicity in humans

Throughout this chapter, the term “dioxins” is used to cover the 29 toxic PCDDs, PCDFs and dioxin-like PCBs discussed in chapter 2.2.1 under *Dioxins and dioxin-like activators*.

Health effects of dioxins. Knowledge of the health effects of dioxins in humans is much less comprehensive than the information available from experimental animals. Epidemiological data that offer insights into the toxic effects in humans are mainly from populations that have been exposed to accidental, major industrial releases of dioxins. The largest release occurred in Seveso, Italy, in 1976, when an industrial plant exploded, releasing about 6 tonnes of various chemicals, including an estimated 2–20 kg of TCDD. The most severely affected population comprised about 17 000 people. In addition to the information from the Seveso and other, smaller epidemiological cohorts, there are also a few case reports of individuals and populations that have been exposed to particularly substantial amounts of TCDD and other dioxins due to accidents, occupational exposure or deliberate poisonings (Geusau *et al.* 2001, Guo *et al.* 2004, Masuda 2001, Sorg *et al.* 2009, S. -. Wang *et al.* 2008).

Unlike in many laboratory animal species, lethality has not been observed in humans, even after high exposures, and overall, humans appear relatively resistant to

the effects of dioxins (Aylward *et al.* 1996, Black *et al.* 2012). This is probably, at least in part, due to their relatively low binding affinity to the human AHR compared with rodents (Okey *et al.* 1994). The highest dose of TCDD measured in a human has been a calculated dose of 25 μg/kg (Geusau *et al.* 2001). Initially, this individual exhibited nonspecific gastrointestinal symptoms, a moderate elevation of blood lipids, leukocytosis, anaemia and secondary amenorrhoea. The only marked toxic effect observed in up to two years of follow-up was severe, generalized chloracne, which is a skin disease and a characteristic adverse effect in humans after very high exposures to dioxins (Geusau *et al.* 2001, White *et al.* 2011). However, based on epidemiological data from the Seveso and other cohorts, other health effects following high exposures have been reported after an extended follow-up.

With very high exposure levels, an association with an overall elevation in cancer risk has been established (White *et al.* 2011). The IARC has also classified TCDD as carcinogenic to humans (Group 1), while other dioxins have been categorised as not classifiable (Group 3) due to inadequate data (International Agency for Research on Cancer 1997). In addition to cancer, high doses of dioxins have been identified to have endocrine disrupting properties within sensitive time windows, and to cause developmental effects, such as an altered sex ratio of offspring, altered sperm quality and disturbances in the development of teeth, including hypomineralisation and hypodontia (Alaluusua *et al.* 1996, Alaluusua *et al.* 2004, Viluksela *et al.* 2011, White *et al.* 2011).

Less evident, but still possible adverse health effects in humans include type 2 diabetes and reproductive effects, for instance an increased risk of infertility. While effects on blood lipids, thyroid function and cardiovascular health are seen in experimental animals, the epidemiological data for these effects in humans remain conflicting. Also, based on the epidemiological data, it is unclear whether dioxins have immunological effects in humans. Some of the epidemiological cohorts are still being followed, and upcoming data may provide more information on these so far ambiguous effects.

TEF/TEQ concept. In the environment, TCDD is always present in mixtures, and it is therefore not generally possible to directly assess its effects in humans. Furthermore, as dioxins are a group of structurally related but toxicologically different contaminants, their risk assessment should reflect this to be effective. Hence, instead of the assessment of individual congeners, a different approach is taken. Based on current knowledge of the compounds, a concept of toxic equivalency (TEQ) is used to report toxicity-weighed masses of dioxin mixtures (Van Den Berg *et al.* 1998). This allows a comparison of the toxicity of different congeners, as well as an assessment of their combined effects.

The approach is based on the chemical structures and AHR-binding abilities of different congeners, their toxic potencies, persistence in the environment and accumulation within organisms and in the food chain, and the general consensus that

the toxic effects of dioxins are mediated by the AHR and are thus additive. Therefore, each dioxin congener has been assigned a toxic equivalency factor (TEF) by the World Health Organization (WHO) to reflect their toxicity in relation to that of TCDD (Van den Berg *et al.* 2006). TEFs are further used for the calculation of total mixture TEQs by multiplying the mass of each congener by its TEF, and then summing the result for all the congeners in a mixture. The TEFs undergo re-evaluations as additional data are obtained. However, it is recognized that while their use is generally accepted, TEFs are of necessity approximations based on incomplete data (Gasiewicz and Henry 2011) and this approach therefore has limitations.

Risk assessment of dioxins. Food is the primary human source of dioxins, which are particularly present in fatty animal products such as dairy products, eggs and meat (Scientific Committee on Food 2000). This is typically a result of the bioaccumulation of dioxins in the food chain, but there have also been several cases of dioxin contamination in farmed animal feed, leading to the resulting foodstuffs containing high levels of dioxins. In the Nordic countries, the main source is fatty Baltic Sea fish, especially Baltic herring and salmon, which contain relatively high (although declining) concentrations of dioxins (Hallikainen *et al.* 2011). The risk groups for dioxin exposure are considered to be children and thus also women of childbearing age, due to susceptibility to the effects of dioxin during development. Excluding accidental exposures that are not typically relevant to the general public, the estimated average intake of dioxins in many countries is below 5 pg/kg BW/day (Bilau *et al.* 2008, Fromme *et al.* 2009, Safe *et al.* 2011). However, the amounts can be significantly higher for individuals consuming large quantities of products with high dioxin levels.

The former European Commission Scientific Committee on Food set the tolerable weekly intake for dioxins at 14 pg WHO TEQ/kg BW (Scientific Committee on Food 2001). The Joint Expert Committee on Food Additives of WHO and the UN Food and Agriculture Organisation similarly established a tolerable monthly intake at 70 pg/kg BW for dioxins (Joint FAO/WHO Expert Committee on Food Additives 2002). However, a more recent risk assessment by the US Environment Protection Agency concluded on a lower dose level, denoting an estimate of the likely lifelong safe exposure, at 0.7 pg/kg BW/day (United States Environmental Protection Agency 2012). An updated risk assessment of dioxins by the European Food Safety Authority is currently in progress.

A major problem with dioxin risk assessment is the large variation in dioxin effects between and within species, which complicates extrapolation from *in vivo* studies to human health hazard and risk assessment. This holds especially true without robust mechanistic information from each species. Therefore, the need to better understand the molecular mechanisms of both physiological and dioxin-related AHR pathways, and differences among species and different developmental stages, is also valuable for risk assessment.

2.3.2 Physiological functions of the AHR

The physiological functions of the AHR are far less understood than the dioxin-induced toxicological effects. This is due to research efforts directed towards physiology having increased much later than those concerning the structure of the protein and its toxicological effects, where the research started. The lack of evident endogenous modulators further contributed to this. More recently, however, instead of considering the AHR as chiefly important in toxicology, the view has shifted towards seeing AHR-mediated toxicity as a result of over-expression or dysregulation of its primary physiological functions (Kung *et al.* 2009).

Nonetheless, the notion that the AHR is likely to have physiological effects has prevailed almost as long as the receptor has been known to exist. One of the early findings in this field was that there are significant differences in the health, fertility and life span of mice that are differently sensitive to TCDD (Gasiewicz and Henry 2011, Nebert *et al.* 1984, Nebert 1989). The mice that are more sensitive to TCDD, due to differences in the structure of their AHR homologue, and thus possess a "high-affinity" receptor, have a longer life span than those that are more resistant and have a "low-affinity" receptor, implying that a properly functioning AHR is important for physiology.

The development of several transgenic AHRKO mice models as well as mice with a constitutively active AHR have been important; they have provided evidence that the AHR is involved in numerous physiological functions (Table 2), even if the mechanisms are often currently incompletely understood. However, the different KO mouse strains display slightly differing phenotypes, and the phenotype of KO rats also differs from that of KO mice (Harrill *et al.* 2013, Lahvis and Bradfield 1998).

The physiological function of the AHR that has been recognised longest and is understood in most detail is its participation in xenobiotic metabolism. Furthermore, it appears that the AHR has particularly important functions in foetal development and immunomodulation.

Table 2. Examples of the physiological functions of the AHR in rodents.

Participates in	Examples	References
Metabolism of xenobiotics	Induction of phase I and II enzymes, *e.g.* cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT) enzymes	Reviewed in (Köhle and Bock 2007)
Development	Cell differentiation	(Casado *et al.* 2010, Van Den Bogaard *et al.* 2015)
	Foetal liver development Liver angiogenesis	(Bunger *et al.* 2008, J. V. Schmidt *et al.* 1996)
Reproduction	Development and function of reproductive systems Regulation of female and male fertility	Reviewed in (Karman *et al.* 2011)
Cell growth	Haematopoietic stem cell maintenance Regulation of the cell cycle Regulation of apoptosis	(C. Ko and Puga 2017, Pääjarvi *et al.* 2005, Park *et al.* 2005, K. P. Singh *et al.* 2009)
Immunomodulation (regulating immune and autoimmune function)	Regulation of B and T cell development Induction of apoptosis in T cells in the EAE model (in concert with ER)	(De Abrew *et al.* 2010, Esser *et al.* 2009, Quintana *et al.* 2008, N. P. Singh *et al.* 2007).
	Tumour suppression	Reviewed in (Bersten *et al.* 2013, Fujii-Kuriyama and Kawajiri 2010)
	Maintenance of gastrointestinal homeostasis and protection against intestinal bacterial infections through innate lymphoid cells	(Kiss *et al.* 2011, J. S. Lee *et al.* 2012)
	Sensing of bacterial virulence factors, controlling of antibacterial responses	(Moura-Alves *et al.* 2014)
Control of intestinal microbiota	Moderation of host–microbiota communication	Reviewed in (L. Zhang *et al.* 2017)
Lipid metabolism	Regulation of cholesterol synthesis Regulation of fatty acid synthesis	(J. H. Lee *et al.* 2010, Tanos, Patel *et al.* 2012, Tanos, Murray *et al.* 2012)
Energy balance	Regulation of body weight	(Moyer *et al.* 2016, Moyer *et al.* 2017, Pohjanvirta 2017)

Xenobiotic metabolising enzyme induction. Within organisms, the induction of xenobiotic metabolising enzymes is the most important mechanism by which the metabolism of xenobiotics is regulated. It is typically controlled by a diverse set of ligand-activated transcription factors, including the AHR and several nuclear receptors. As a consequence, the metabolism of chemicals, including drug compounds, is enhanced, which generally renders them easier to excrete from the body. Overall, an enhanced metabolic rate is mainly considered advantageous to an organism, as it augments its detoxification capacity (Nebert *et al.* 2004). However, the induction of metabolism may also lead to toxic consequences such as tissue damage or tumorigenesis. Mechanisms include the bioactivation of potentially

genotoxic xenobiotics such as PAHs, DNA adduct formation and the production of reactive oxygen species, which may consequently lead to oxidative DNA damage (Gasiewicz and Henry 2011, Shimada and Fujii-Kuriyama 2004).

The AHR gene battery includes xenobiotic metabolising enzyme genes whose expression is induced through the activation of the AHR. They encode such enzymes as CYP1A1, CYP1A2, CYP1B1, glutathione *S*-transferase (GST) A1, NAD(P)H quinone dehydrogenase 1 (NQO1) and UDP-glucuronosyltransferase (UGT) 1A (Q. Ma 2011). Of these, the induced transcription of CYP1A1 mRNA is particularly noteworthy, as the molecular mechanism is well understood (Figure 1), and its induction is a fairly rapid and highly sensitive, although not strictly specific, marker for AHR activation (Abraham *et al.* 1988, Hu *et al.* 2007). Unlike what has been presumed for a long time, it appears that the induction of xenobiotic metabolising enzymes, such as CYP1A1, does not automatically indicate dioxin-like toxicity, or correlate with it (Hu *et al.* 2007, Pohjanvirta *et al.* 2011). For instance, AHR-mediated toxicity can be present when CYP1A1 expression has been eliminated (Carney *et al.* 2004), and substantial CYP1A1 induction can be observed without toxicity (Gasiewicz, Rucci *et al.* 1986).

In addition to xenobiotic metabolism, an important function of the CYP1A1 enzyme appears to be autoregulation of AHR activity via a feedback mechanism, attempting to ensure adequate expression of the AHR (Chiaro *et al.* 2007, Q. Ma 2011). Disruption of this autoregulation may lead to toxic outcomes due to both insufficient CYP1A1 metabolism, which may lead to over-stimulation of the AHR, and over-expression of CYP1A1, eventually leading to AHR deficiency.

Over-stimulation of the AHR can result, for instance, from the delayed metabolism of endogenous AHR-activators. Several of them, such as FICZ, are very potent, but have a short duration of action due to efficient metabolism, mainly by CYP1A1 (A. Rannug *et al.* 1987, Wei *et al.* 1998, Wei *et al.* 2000, Wincent *et al.* 2009). Thus, factors that inhibit CYP1A1 activity, such as certain metal ions, drug compounds and polyphenols, may prolong the effect of the endogenous AHR activators, resulting in increased (secondary) AHR activity (Wincent *et al.* 2012). On the other hand, prolonged expression of *Cyp1a1* can deplete the reservoir of endogenous AHR ligands due to their exaggerated metabolism, resulting in an AHR-deficient-like state (Schiering *et al.* 2017). This has been shown by Schiering and colleagues to result in increased susceptibility to enteric infection due to the disrupted function of the immune system. Additional consequences of elevated CYP1A1 activity include changes in the metabolism of a variety of endogenous molecules with signalling properties, such as retinoids (Lampen *et al.* 2000, Shmarakov 2015), steroid hormones (Spink *et al.* 1992), and polyunsaturated fatty acids (Hankinson 2016).

Immunomodulation. The AHR has been identified as part of the molecular pathways of physiological immune responses, and is thus also a target for immunomodulatory therapies (Zhu *et al.* 2014). The AHR is involved in both innate

and adaptive immunity, and has pro- and anti-inflammatory effects, although the mechanisms are not clearly understood at present (Stockinger *et al.* 2014). Furthermore, the AHR may be deeply involved in autoimmunity, as it is highly expressed in Th17 cells (Veldhoen *et al.* 2008), a subset of pro-inflammatory T helper cells that, among other functions, are important in the pathogenesis of autoimmune diseases (Zambrano-Zaragoza *et al.* 2014). Moreover, the AHR has been shown to regulate the differentiation of Th17 cells (Kimura *et al.* 2008, Quintana *et al.* 2008, Veldhoen *et al.* 2008).

In addition to its immunotoxic effects, even TCDD also induces potentially beneficial modulation of the immune system, namely its suppression. This is manifested as the suppression of autoimmune diseases in animal models, including onset of type 1 diabetes (Kerkvliet *et al.* 2009), EAE (Quintana *et al.* 2008), and the lessening of allergic responses (Luebke *et al.* 2001, Schulz *et al.* 2013, Xu *et al.* 2015). In addition, immunomodulation through the activation of the AHR by other agonists has also been shown to diminish the severity of numerous inflammatory conditions, such as EAE (Quintana *et al.* 2010), colitis (Arsenescu *et al.* 2011, Benson and Shepherd 2011, Furumatsu *et al.* 2011, Goettel *et al.* 2016) and psoriasis (Di Meglio *et al.* 2014). Furthermore, the AHR has been shown to sense and bind distinct, pigmented bacterial virulence factors, and to subsequently control antibacterial responses (Moura-Alves *et al.* 2014).

It has been proposed that non-canonical AHR signalling could be involved in these immunomodulatory processes, for instance through cross-talk with NF-κB signalling (Gasiewicz and Henry 2011, Matsumura 2009, Stockinger *et al.* 2014). Interestingly, several flavonoids and other types of polyphenols, some of which were described under *Dietary AHR modulators* in chapter 2.2.1, have also been shown to have significant anti-inflammatory activity (González *et al.* 2011). Furthermore, even though the group of polyphenols includes compounds of very diverse structures, their effects on inflammation appear highly consistent, involving, for instance, the inhibition of NF-κB signalling.

Anti-tumorigenicity. Furthermore, it appears that the AHR can act in antitumour pathways (Bersten *et al.* 2013). As already briefly discussed in chapter 2.2.1 under *Selective modulators (SAHRMs)*, AHR modulators can have protective, tumour-suppressive properties, especially towards hormone-related cancers such as breast and prostate cancers. It was already reported in the 1970s that while increasing the incidence of some types of tumours, TCDD as a chronic treatment also seemed to protect female rats from spontaneous mammary gland and uterine tumour formation (Kociba *et al.* 1978). This appears to be related to crosstalk between the AHR and ER signalling pathways (Gasiewicz and Henry 2011, Safe and Wormke 2003). Activation of the AHR has later been shown to inhibit invasive and metastatic activities in both ER-dependent and -independent human breast cancer cell lines, and in breast cancer stem-like cells (Gasiewicz and Henry 2011, Hall *et al.* 2010, Prud'Homme *et al.* 2010).

There are further results, particularly from AHRKO mouse models, suggesting antitumorigenic roles for the AHR through several possible mechanisms (Bersten *et al.* 2013, Ohtake and Kato 2011), including antiproliferative regulation via non-canonical signalling and anti-inflammatory mechanisms mediated by the AHR. As discussed in chapter 2.2.1 under *Selective modulators (SAHRMs)* and *Pharmaceuticals*, there are already compounds on the market used for anticancer therapy that have later also been shown to modulate AHR activity.

2.4 TCDD and novel food avoidance behaviour

One of the more peculiar responses to TCDD exposure observed in rats and mice is novel food avoidance behaviour. An originally fortuitous and unexpected finding revealed that low, well below acutely toxic doses of TCDD result in a strong and very persistent avoidance of previously unfamiliar foodstuffs when administered either simultaneously with or a short time before the introduction of the foods (Lensu *et al.* 2011a, Lensu *et al.* 2011b, J. T. Tuomisto *et al.* 2000).

Interestingly, the well-documented rat strain differences in sensitivity to TCDD are not reflected in susceptibility to this behaviour; both TCDD-sensitive and -resistant rat lines exhibit comparable avoidance behaviour (Lensu *et al.* 2011b). It is displayed towards different types of novel foodstuffs, including milk chocolate, cheese, and 10% sucrose and 0.25% saccharin solutions. Even a change in the texture of the standard feed (pelleted vs. powdered) has been found sufficient to induce the avoidance (Lensu *et al.* 2011a). Furthermore, unlike many other TCDD-induced effects, the avoidance response emerges rapidly, within hours of a single TCDD exposure coupled with the presentation of a novel food. Moreover, it emerges at very low doses of ≤ 1 μg/kg, in both TCDD-sensitive and -resistant rat lines (Lensu *et al.* 2011b). Hence, this response is one of the most sensitive behavioural effects TCDD has been shown to exert in adult laboratory animals.

The avoidance behaviour appears to closely resemble two behavioural phenomena: taste neophobia and conditioned taste avoidance (CTA; often also used interchangeably with the term conditioned taste aversion). Both of them occur in animals and humans alike. They are behavioural mechanisms that have been interpreted to have evolved to protect animals from eating potentially harmful food. It has also been suggested that taste neophobia and CTA can be intertwined, so that when suspicions of toxicity are aroused, the former mechanism primes the latter to become engaged and enhanced (Lin *et al.* 2016).

Taste neophobia. Neophobia is considered an innate, protective behaviour that can be experienced towards food, but also novel objects or environments (Corey 1978). Taste neophobia is a novelty-induced fear response that prevents the ingestion of large amounts of novel foods, and typically subsides rapidly once the novelty becomes

familiar and is deemed safe. Like CTA, it appears to involve aversive behaviour, whereby the palatability of the novel food is initially reduced (Lin *et al.* 2012). Taste neophobia is particularly strong in rodents, and is a beneficial trait, as they are omnivores but unable to vomit.

Conditioned taste avoidance. CTA is considered a special form of classical conditioning, where avoidance develops towards the taste or odour of a specific foodstuff (conditioned stimulus, CS) and is paired with an unconditioned stimulus (US) that is experienced in conjunction with, or relatively soon after, consuming the food (Lin *et al.* 2016, Verendeev and Riley 2012, Welzl *et al.* 2001). This behavioural change prevents the animal from further ingesting a food deemed harmful after its consumption, regardless of whether the CS and the US are in reality causally related. Unlike taste neophobia, CTA may develop towards familiar foods and even persist for weeks or months.

Classically, the US has been considered to be nausea or gastrointestinal malaise. However, CTA has also been repeatedly described following treatments that do not induce vomiting in species that are capable of it, and therefore it appears that the feeling of nausea or gastrointestinal discomfort is not always required for the effect to take place. In fact, CTA can also be instigated when animals are unconscious while exposed to CTA-inducing compounds, and likewise by drugs that are rewarding, such as amphetamine (Grant 1987, Lin *et al.* 2012, Lin *et al.* 2014, Verendeev and Riley 2012).

Therefore, it has been suggested that there could be two distinct processes behind CTA, conditioned taste aversion and conditioned taste avoidance (Parker 2003). This distinction proposes aversion to be displayed by active rejection reactions such as gapes, chin rubs and paw treads, and avoidance by reduced appetite and consumption in the absence of active rejection. The measurement of taste reactivity, particularly by analysis of lick patterns, is also more generally considered essential in the characterisation of CTA, as it mirrors palatability and reveals active rejection (Arthurs *et al.* 2012, Lin *et al.* 2017, Lin *et al.* 2012). Furthermore, it has been proposed that nausea or gastrointestinal discomfort would only be a prerequisite for aversion, while avoidance could be related, for instance, to conditioned fear motivated by changes in the physiological state in general (Parker 2003). However, there are experimental results showing that several compounds not inducing nausea produce aversion as analysed by lick patterns (Arthurs *et al.* 2012, Lin *et al.* 2017, Lin *et al.* 2012). Therefore, further studies are warranted in order to more precisely define CTA. In this thesis research, CTA was considered as a general conditioned taste avoidance behaviour, as the assessment of palatability was not possible in the studies performed.

Studies with TCDD have provided support for the involvement of both taste neophobia and CTA, as elements of both appear to be involved. The finding that chocolate avoidance in rats could be elicited even if chocolate was first presented as

long as one day after TCDD exposure (Lensu *et al.* 2011a) strongly argues for enhanced neophobia. For CTA to occur, it is considered that the CS and the US should be temporally close together. A number of other facts attest to the involvement of CTA. Firstly, clear avoidance also emerges, albeit not quite as strikingly, when rats have been accustomed to chocolate for a few days or up to a month prior to TCDD exposure (Lensu *et al.* 2011a, J. T. Tuomisto *et al.* 2000). Secondly, Tuomisto and colleagues (2000) showed that rats that had been given chocolate within 12 h of TCDD exposure consumed cheese (which at that point was a novel food item for them) on day 13 post-exposure equally well compared to the control group. However, these same rats still demonstrated chocolate avoidance on day 19 post-exposure. And finally, a parallel finding was reported by Lensu and colleagues (2011a) following an experiment in which rats were exposed to TCDD and offered an unfamiliar food (chocolate or powdered chow), which was subsequently available for one day. Seven days after the TCDD exposure coupled with the novel food, the rats preferred a completely novel food (powdered chow or chocolate) to the one they had been offered as unfamiliar on the day of exposure.

Two further effects that could be related to novel food avoidance behaviour are hypophagia and anxiety. However, TCDD doses that induce novel food avoidance are very low, far below those causing wasting syndrome, and do not have an effect on the intake of familiar chow (Lensu *et al.* 2011a). As for anxiety, there are established tests in which an increased latency to consume familiar foods in a novel environment (hyponeophagia) is indicative of heightened anxiety (Dulawa 2009, Samuels and Hen 2011). However, a study on TCDD-resistant H/W rats found no indication of heightened anxiety after TCDD treatment, even at very high doses, which for most rat strains would have been lethal (Sirkka *et al.* 1992). While these rats are resistant to the lethality of TCDD, they are equal to other strains in their sensitivity to most biochemical and behavioural responses measured, including novel food avoidance behaviour (Lensu *et al.* 2011b).

3 AIMS OF THE STUDY

The AHR has long been acknowledged as the key mediator in dioxin-induced toxicity. In addition, it has important physiological functions. However, knowledge of both of these, as well as the underlying mechanisms, is still incomplete. The objective of this study was to contribute to the elucidation of some of the many roles of the AHR. The overall aim was two-fold: to study the effects of two novel SAHRMs and their respective pro-drugs, and to further examine the novel food avoidance behaviour previously characterised following TCDD exposure.

The specific aims of this thesis research were to:

1) Characterise selected *in vitro* effects of the active SAHRMs C1 and C3, intended as novel drug compounds for the treatment of AHR-related maladies (III);

2) Characterise the acute and subacute *in vivo* toxicological effects of the prodrug-SAHRMs C2 and C4 (II);

3) Find out the extent to which the effects of these novel compounds resemble or differ from those of TCDD (I–III);

4) Determine whether novel food avoidance behaviour is AHR-dependent in rats (I, unpublished);

5) Further elucidate the mechanisms behind the behaviour and its possible connection to CTA, a known behavioural change considered as a special form of classical conditioning (I, unpublished).

4 MATERIALS AND METHODS

4.1 Chemicals (I–III, unpublished)

Selective AHR modulators used *in vitro* and *in vivo* (I–III). Novel SAHRMs C1, C2, C3 and C4 (Figure 8; Table 3) were studied for their selected toxicological and physiological properties *in vitro* and *in vivo*. The compounds were kindly provided by Dr Lars Pettersson (Immunahr AB, Lund, Sweden).

C1 and C3 are AHR-active *N*-hydrogen metabolites of the immunomodulatory drug compounds laquinimod and tasquinimod. While C1 and C3 can be used as such *in vitro*, they are unsuitable for *in vivo* formulations due to their low aqueous solubility. Thus, *in vivo*, the respective acetylated prodrugs C2 and C4 are used, as they are readily hydrolysed by xenobiotic metabolism to provide the active compounds C1 and C3.

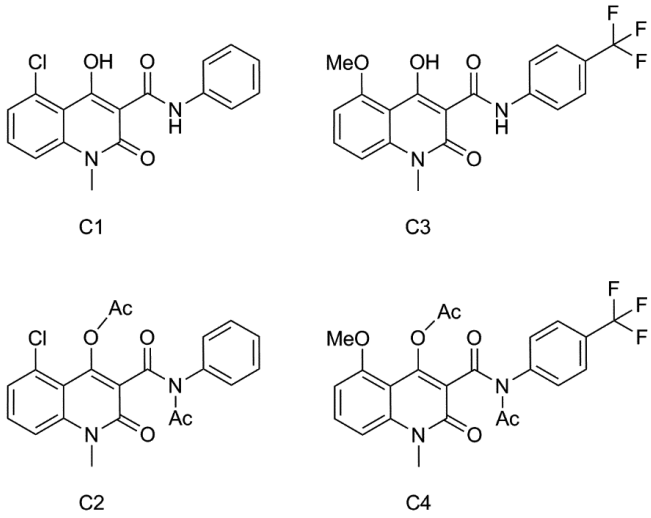


Figure 8. Chemical structures of the novel selective AHR modulators

In the *in vitro* experiments, C1 and C3 were studied for their toxicity and ability to activate the AHR. Both were dissolved in DMSO (Sigma-Aldrich), and dissolution of the high concentration stocks was aided by heating in water baths at +37–65 °C or +65–85 °C, respectively, for 30–60 min. For the mammalian cell line experiments, the DMSO solutions were further diluted with culture medium before application to cells (DMSO concentration applied to cells did not exceed 0.1%).

In vivo, C2 and C4 were studied for their toxicity and ability to induce novel food avoidance. Stock solutions were prepared by mixing the compounds with PEG-400 (Ph. Eur. grade, Sigma-Aldrich, St. Louis MO, USA) and heating in a water bath at +80–95 °C for 30–60 min, with intermittent vortexing to aid dissolution. Further dilutions were prepared from the stocks with PEG-400.

Table 3. Identification information for the novel selective AHR modulators

Abbreviation	Company code	Chemical name	CAS registry number
C1	IMA-06201	N-ethyl-N-phenyl-5-chloro-1,2-dihydro-4-hydroxy-1-methyl-2-oxo-quinoline-3-carboxamide	879410-94-3
C2	IMA-08401	N-acetyl-N-phenyl-4-acetoxy-5-chloro-1,2-dihydro-1-methyl-2-oxo-quinoline-3-carboxamide	1373260-17-3
C3	IMA-06504	N-(4-trifluoromethylphenyl)-1,2-dihydro-4-hydroxy-5-methoxy-1-methyl-2-oxo-quinoline-3-carboxamide	1373259-57-4
C4	IMA-07101	N-acetyl-N-(4-trifluoromethylphenyl)-4-acetoxy-1,2-dihydro-5-methoxy-1-methyl-2-oxo-quinoline-3-carboxamide	1373259-76-7

Other test compounds used *in vitro* (II, III). In addition to examining the effects of C1 and C3, they were compared with those of TCDD, when relevant. Furthermore, the ability of CH-223191 (Sigma-Aldrich, St. Louis MO, USA) to antagonise C1 and TCDD was tested. TCDD was purchased from Ufa Institute (Ufa, Russia) and was over 98% pure, as assessed by gas chromatography–mass spectrometry. Stock solutions of CH-223191 and TCDD were prepared by dissolving them in DMSO (Sigma-Aldrich). The stocks were further diluted with cell culture medium before application to cells (maximum DMSO concentration applied to cells was 0.1%).

Chemicals used as positive controls included Triton X, BaP, sodium azide, 2-aminoanthracene, and mitomycin c (all from Sigma-Aldrich). Apart from Triton X, they were dissolved in either H_2O or DMSO (final DMSO concentration on H4IIE cells was 0.1%, and 1.85% in the Ames test on *Salmonella* Typhimurium cells).

Other test compounds used *in vivo* (I, unpublished). For the novel food avoidance behaviour experiments, AHR agonists FICZ, BNF and BaP were used in addition to C2. Furthermore, a constitutive androstane receptor (CAR) agonist, TPD, was used to study whether phenobarbital-induced xenobiotic metabolising enzyme activation, following the induction of CAR instead of AHR, would lead to similar novel

food avoidance behaviour. AHR antagonists CH-223191 and GNF351 were used to test whether the avoidance response could be alleviated.

FICZ was purchased from Enzo Life Sciences (Farmingdale NY, USA) and BNF, BaP and CH-223191 from Sigma-Aldrich. GNF351 Calbiochem® was from Merck (Darmstadt, Germany). TPD was courtesy of Dr Vladimir O. Pustylnyak (Institute of Molecular Biology and Biophysics SB RAMS, Novosibirsk, Russia).

FICZ, TPD, CH-223191 and GNF351 were first dissolved in DMSO and then diluted with sunflower oil [Keiju, Bunge Finland Oy, Raisio, Finland; final DMSO concentrations 2.5% or 6% (v/v)]. BNF and BaP were mixed with either DMSO and sunflower oil or just sunflower oil and heated in a water bath at +95 °C for 1 h, resulting in suspensions [final DMSO concentrations 2.5% (v/v)].

4.2 H4IIE cell line (II, III)

The H4IIE rat hepatoma cell line (H-4-II-E ATCC® CRL1548™) was acquired from ATCC (Manassas VA, USA). The cell line was selected for its highly inducible expression of CYP xenobiotic metabolising enzymes (Fujimura *et al.* 2012). In addition, it has been shown to be exceptionally responsive to CYP1A1 induction by dioxins (Bradlaw and Casterline Jr. 1979, Sawyer and Safe 1982).

The cells were cultured in monolayers at 37 °C and 5% CO_2 in low glucose Eagle's Minimum Essential Medium (EMEM; ATCC) or low glucose Dulbecco's Modified Eagle Medium (Gibco® DMEM, Thermo Fisher Scientific, Paisley, Scotland, UK), depending on the following experiment. Both media were supplemented with 10% FBS (Sigma-Aldrich) for cell culturing. Cell passages from 2 to 6 were used for the experiments. The MycoAlert™ Mycoplasma Detection Kit (Lonza Group Ltd, Basel, Switzerland) was periodically used to ensure that the cells used in the experiments were free of infection.

4.3 Animals and their husbandry (I, II, unpublished)

For I, II and an unpublished experiment, male Sprague Dawley (SD) rats were purchased from Harlan Netherlands. For the unpublished experiment, the rats were vagotomised. The operations were performed at Harlan, allowing for recovery before shipment. In addition, for another unpublished experiment, AHR-knock-out SD rats (AHRKO) were bred in the laboratory animal centre of the University of Helsinki. For this purpose, a pair of homozygous AHRKO rats (SD-Ahr^{tm1sage}) was acquired from Horizon Discovery (Waterbeach, Cambridge, UK). This rat strain, originating from wild-type (WT) SD rats, harbours a deletion mutation of 760 base pairs in exon 2, which contains the DNA-binding bHLH motif of the AHR gene.

In order to establish a specific pathogen-free colony, the KO male was paired with a pathogen-free, superovulated WT SD female (from Harlan). Subsequently, the embryos were transferred to a pseudopregnant recipient female. The resulting heterozygote offspring consisting of three females were paired with a WT male (Harlan). Subsequent heterozygote progeny were further paired with each other (avoiding littermate pairings) to gain homozygote AHRKO and littermate WT rats for use in experiments. Genotyping of the rats was performed by PCR (details in chapter 4.17) using gDNA extracted from ear punches.

All rats were acclimatised to the study conditions and handling for a minimum of one week before commencing the experiments. Throughout the studies, they were housed in individually ventilated plastic cages (Sealsafe IVC Blue Line or Green Line IVC Sealsafe PLUS Rat, Techniplast, West Chester PA, USA), and maintained on a 12-h light/dark cycle (06:00–18:00). The cage floor was covered with aspen wood bedding (Tapvei, Estonia), and each cage was enriched with a transparent red plastic hiding tube, nesting material and chew blocks (both aspen wood, Tapvei, Estonia). Commercial pelleted rat chow [RM1 (E) SQC Expanded, SDS Diets, Witham, Essex, England; or equivalent Teklad Global 16% Protein Rodent Diet, Teklad Diets, Madison WI, USA] and filtered, UV-irradiated tap water were available *ad libitum*. The animal room was air-conditioned: the temperature was kept at 22 ± 1 °C and the relative humidity at 38–75% (typically 50%).

Ethical issues (I, II, unpublished). *In vivo* studies were authorized by the National Animal Experiment Board in Finland (Eläinkoelautakunta, ELLA; project licence code ESAVI/6882/04.10.03/2012). All procedures were conducted in a humane manner and in accordance with Directive 2010/63/EU of the European Parliament and of the Council.

4.4 Cytotoxicity assay (III)

The cytotoxicity of C1, C3 and TCDD was assessed in H4IIE cells using the Cytotoxicity Detection Kit (LDH; Roche Diagnostics GmbH, Mannheim, Germany), which measures lactate dehydrogenase (LDH) leakage. A total of 25,000 cells were seeded per well in 96-well plates (Greiner Bio-One GmbH, Kremsmünster, Austria) ~18 h prior to exposures. The outer and corner wells were left empty of cells and only filled with PBS to avoid the edge effect. As recommended in the manual, the medium (EMEM) was supplemented with 1% FBS for the assay to prevent LDH in serum from increasing the background absorbance. The concentrations of the test compounds used were 10, 100 and 1000 nM for C1 and C3, and 10 and 100 nM for TCDD. Cells were exposed for 6 h and 24 h. The vehicle was used as a negative control (0.1% DMSO) and Triton X-100 (1%) as a positive control. Background controls (assay medium without cells, with and without vehicle) were also included. All exposures

were performed in triplicate in two independent experiments. Culture supernatants were collected, and the assay was performed according to the manufacturer's instructions. Absorbances were measured with an ELISA reader at 492 and 620 nm and corrected for the measured background absorption (Multiskan Ascent, Thermo Fisher Scientific). Cytotoxicity was calculated as a percentage using the following equation: [(exp. value - negative control) / (positive control - negative control) * 100]. The negative control was thus at 0% cytotoxicity and the positive control at 100%, and the cytotoxicity of the test compounds expressed relative to these.

4.5 MTT reduction assay (III)

The effect of C1, C3 and TCDD on cell viability/metabolic activity *in vitro* was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay in H4IIE cells. The colorimetric MTT assay is based on the information that viable cells with active metabolism convert the yellow tetrazolium dye MTT into a purple formazan product, which can be measured. When cells die, they can no longer perform this conversion. The molecular pathways involved in disturbances in MTT reduction into formazan are not well understood, but in controlled study conditions, the amount of formazan product formed by NAD(P)H-dependent cellular oxidoreductase enzymes is in proportion to the number of metabolically active, viable cells (Riss *et al.* 2013 (Updated 2016)). Therefore, and as the data from the test compounds were compared with the vehicle controls (100% viability and metabolic activity), the result was interpreted to reflect the reduction in metabolic activity.

Altogether, 24,000 H4IIE cells were seeded per well in 96-well plates (Greiner Bio-One GmbH) 24 h prior to exposures. The outer and corner wells were only filled with PBS to avoid the edge effect. The medium used in the assay was low-glucose, phenol red-free DMEM (Gibco® DMEM, Thermo Fisher Scientific, Paisley, Scotland, UK) supplemented with 4 mM L-glutamine (Thermo Fisher Scientific), corresponding to the standard DMEM used for culturing the cells prior to the experiment. The medium used in the experiment was further supplemented with 1% FBS (Sigma-Aldrich).

The cells were exposed to the vehicle, C1, C3 or TCDD for 6 h or 24 h at 5–6 concentrations (1, 10, 50, 100, 500 and 1000 nM of C1 and C3 on cells; 1, 5, 10, 50 and 100 nM of TCDD). All exposures were performed in triplicate in two independent experiments. After exposures, the cells were washed once with PBS and treated with MTT for 5 h (Sigma-Aldrich; final concentration 1 mg/ml). The medium was then gently removed, and formazan crystals were dissolved in 200 µl of DMSO containing 0.1 M glycerine and 0.1 M NaCl. After incubation at room temperature for 10 mins, the absorbances were measured with an ELISA reader at 595 nm (Multiskan Ascent,

Thermo Fisher Scientific). The results were calculated as a percentage over the controls.

4.6 CYP1A1 induction assay (II, III)

The CYP1A1 enzyme induction potential of C1, C3 and TCDD was tested *in vitro* in the metabolically active H4IIE cell line using a luminescent assay. CYP1A1 induction was utilised to represent AHR activation in general, as it is a well-established, fairly rapid and highly sensitive index of AHR activation (Abraham *et al.* 1988). The aim was to compare the potency and efficacy of the novel compounds with those of TCDD. In addition, the ability of the specific AHR antagonist CH-223191 to block the effect of C1 and TCDD was tested.

Several independent experiments were performed in 96-well plates (Greiner Bio-One GmbH, Kremsmünster, Austria). Cells were seeded at 10,000 cells/well and allowed to equilibrate for about 40 h prior to exposures. The outer and corner wells were left without cells and filled with PBS in order to avoid the edge effect. The cells were then exposed for 24 or 48 h to varying concentrations of C1, C3 and TCDD (1, 10, 100, 500 pM and 1, 5, 10, 50 and 100 nM, and furthermore 1000 nM of C1 and C3). In addition, a combination of C1 or TCDD and CH-223191 (1 nM and 100 nM, respectively) was used. The controls were exposed to the vehicle (0.1% of DMSO in culture medium). For 48-h exposures, culture medium with the test compounds was replaced with fresh medium at 24 h and the exposures repeated. All exposures were performed in triplicate.

CYP1A1 activity was detected with the P450-Glo™ CYP1A1 Assay (Promega, Madison WI, USA) according to the manufacturer's instructions. Subsequently, the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was used to confirm that there were no significant differences between the numbers of viable cells in the wells at the time of detection. The assay is based on the quantitation of ATP.

4.7 Ames test (III)

The genotoxicity of C1 and C3 was first examined by the standard plate incorporation mutagenicity test, which was performed according to the principle of Maron and Ames (1983). Briefly, TA98 and TA100 *Salmonella* Typhimurium strains were used, and the test was performed both with and without metabolic activation, using 10% S9 SD rat liver mix (Trinova Biochem GmbH, Giessen, Germany). Water and DMSO (1.85% on plates) were used as negative controls for both strains. As positive controls for TA100 and TA98, respectively, sodium azide (0.04 mg/ml) or 2-aminoanthracene (0.2 mg/ml) was used. In addition, BaP (0.1 mg/ml) was used as a positive control for

both strains. The volume of both control and test compounds was 50 µl per plate. Two independent tests were performed. Within each test, triplicate or quadruplicate plates were prepared for each compound and dose, and for all controls. In the first test, C1 and C3 were assayed at concentrations of 0.375, 0.75, 1.5 and 3 mg/ml, resulting in plate concentrations of 17.5–170 µM. In the second test, plate concentrations of 1 nM, 100 nM, 10 µM and 1 mM were used. All plates were incubated at 37 °C for 72 h before calculation of colonies.

4.8 *In vitro* micronucleus test (III)

The Ames test is able to detect only mutagens. Therefore, to further assess any genotoxic properties of C1 and C3, also an *in vitro* micronucleus test, which detects chromosomal aberrations, was performed on the H4IIE cell line. TCDD was included here in addition to C1 and C3.

A total of 40 000 cells were seeded in 12-well plates (Greiner Bio-One GmbH) 24 h prior to exposures. The medium employed was low-glucose, phenol red free DMEM (Gibco® DMEM, Thermo Fisher Scientific) supplemented with 4 mM L-glutamine (Thermo Fisher Scientific) and 10% FBS, corresponding to the standard DMEM used for culturing the cells prior to the experiment. S9 mix was not employed, as the cell line has retained a considerably high metabolic activity (Fujimura *et al.* 2012).

Mitomycin c was used as a positive control, and vehicle as a negative control. The final concentrations on cells were: 100, 500, and 1000 nM of C1 and C3; 10, 50, and 100 nM of TCDD; and 500 nM of mitomycin c. All exposures were performed in triplicate in a single experiment. The exposure time was 24 h, after which the cells were treated with cytochalasin B (4 µg/ml; Cayman Chemical, Ann Arbor, MI USA) for 1.5–2 normal cell cycle lengths, ~28 h. Next, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 10–15 min (in PBS, pH 6.9; Sigma) before washing again with PBS. For detection, the cells were stained with 10% Giemsa solution (Merck KGaA, Darmstadt, Germany) and the micronucleus frequencies were analysed at 1000 binucleated cells per well.

4.9 *In vivo* experimental design

Within all *in vivo* experiments, rats were randomly allocated into groups, which were matched for body weight (BW) and, when necessary, genotype (unpublished experiments with AHRKO rats). In all experiments, rats were weighed immediately before exposures and dosed according to BW. In all novel food avoidance behaviour studies (I, unpublished), rats were housed singly during the experiments to allow the measurement of novel food consumption. In II, rats were housed in groups

throughout the experiments. At the end of the studies, carbon dioxide was used for euthanasia and samples were collected.

4.9.1 Acute and subacute toxicity of the novel SAHRMs (II)

Acute toxicity. The study was carried out on ~9-week-old male SD rats as a pilot experiment. The aim was to ensure that the pro-drugs C2 and C4 would not cause marked acute toxicity before their repeated administration to larger groups of animals. The study principle was loosely based on the OECD test guideline for acute oral toxicity [Up-and-Down-Procedure (OECD 2008)] to reduce the number of animals required.

Single doses at three different dose levels were tested for both compounds, 8.75–92.5 mg/ml for C2 and 8.75–75 mg/ml for C4. The high doses were limited by the maximum achievable solubility of the compounds. The volume administered was 5 ml/kg, and all groups were dosed intragastrically (*ig*) by oral gavage. Post-exposure, the rats were monitored for 7–13 days before necropsy.

As the number of rats in each group was low in this experiment (n = 1–3, except for controls where n = 6), the data from it were considered preliminary, and statistical evaluation was only performed among the controls and the highest doses of C2 and C4 (n = 3).

Subacute toxicity. Likewise, ~9-week-old male SD rats (n = 5–6 in each group) were employed to explore the toxicological properties of C2 and C4 after repeated administrations. The substances were administered once a day on five consecutive days at the highest concentrations attainable, which had proven to not be acutely toxic in the pilot experiment. These were 75 and 100 mg/kg/day for C4 and C2, respectively (5 ml/kg, *ig*). After the last exposure, the rats were further monitored and weighed for five days before necropsy for any delayed clinical signs. At necropsy, body and selected organ weights (thymus, liver, kidneys, testes and spleen) were determined, and serum and tissue samples (liver, duodenum, kidney, testis and lung) were frozen in liquid nitrogen for further processing (chapters 4.10–4.14). In addition, liver, spleen, kidney, lung and testis samples were collected for histopathology (chapter 4.13).

4.9.2 Novel food avoidance behaviour studies (I, unpublished)

Novel food avoidance behaviour was studied in several experiments described below. All exposures were performed in the forenoon, between 10:30–12:00. The dosing route in all experiments was either *ig* or *ip*, and the volume dosed was 4 or 5 ml/kg. These were kept constant across groups within experiments, and also between them when possible. Apart from a 3-h fast before *ig* dosing, normal chow was available

concurrently with the novel food throughout the studies. Tissue samples were collected after euthanasia from most studies to determine whether the AHR had been activated (analysed by RT-qPCR as *Cyp1a1* induction).

As in many of the earlier studies with TCDD, the novel food item used in all of the experiments was milk chocolate, which rats typically find highly palatable. In addition, chocolate keeps well at room temperature, and its consumption is easy to measure: the level of quantification is low (down to 10 mg, depending on the sensitivity of the scales used), and discriminating even between a single bite (tasting) and no bite is easy.

Ability of AHR agonists to induce novel food avoidance (I). Previously, novel food avoidance behaviour had only been studied after TCDD exposure (Lensu *et al.* 2011a, Lensu *et al.* 2011b, J. T. Tuomisto *et al.* 2000). In I, the aim was to determine whether the effect is specifically characteristic of TCDD, or whether it can also be induced by other AHR activators. To this end, male SD rats (n = 6/group) were exposed to single *ig* doses of the established AHR agonists FICZ (0.1 mg/kg), BNF (60 mg/kg) and BaP (150 mg/kg). The doses used were set based on previous literature demonstrating that they activate the AHR *in vivo* (Hodek *et al.* 2013, Mukai and Tischkau 2007, Wincent *et al.* 2012). Furthermore, in a separate experiment, single *ig* doses of the SAHRM C2 were tested, similarly in SD male rats, at three dose levels (4, 20 and 100 mg/kg; n = 6/group). In both experiments, chocolate was placed in each cage directly post-exposure, and its consumption measured for a minimum of 24 h, or as long as the avoidance response persisted.

Dependence of the response on the AHR (I, unpublished). Findings in a previous study with TCDD in AHRKO mice had already implied the specific involvement of the AHR in the avoidance response (Lensu *et al.* 2011b). Here, the aim was to confirm in SD rats whether, in addition to a correlation, there is indeed causality between AHR activation and the novel food avoidance behaviour. To this end, in I, alleviation of AHR-activator-induced novel food avoidance was attempted with two different AHR antagonists, CH-223191 and GNF351. They were administered separately *ig* as single doses (13 mg/kg of CH-223191 and 5 mg/kg of GNF351), in addition to single doses of FICZ (0.1 mg/kg, *ig*; n = 6/group). Furthermore, single doses of CH-223191 and C2 were tested: 10 mg/kg, *ip* of the antagonist together with 20 mg/kg, *ig* of C2, and 4 mg/kg, *ig* of C2 together with 15 mg/kg, *ig* of antagonist (n = 6/group).

Moreover, an AHRKO rat line was acquired (details in 4.3), which allowed directly exploring the involvement of the AHR in the response. In an unpublished experiment, it was tested whether BNF (60 mg/kg, 4 ml/kg, *ig*) would induce novel food avoidance behaviour in male AHRKO rats. Two experiments both consisted of four groups: AHRKO and littermate WT BNF groups, and control groups of both genotypes. The first experiment (n = 3/group) was conducted as before, and novel chocolate was

placed in the cages directly after exposures. However, both genotypes of this rat line behaved somewhat differently compared to the Harlan SD rat line used in previous experiments, with controls exhibiting a considerably slower onset of chocolate consumption, possibly reflecting enhanced neophobic behaviour in this strain. Therefore, in the second experiment (n = 6–7/group), the study design differed somewhat from the previous one. Chocolate was employed as the novel food item, as before, but was now placed in each cage for ~24 h prior exposure to BNF. The rationale was to ensure that each rat was familiar with chocolate by exposure, and that it did not exhibit neophobic behaviour. This also allowed approaching the question of the two possible underlying food avoidance phenomena, neophobia and CTA, because for CTA to develop, association of a taste (or odour) with the stimulus is essential, and in classical CTA designs it is typically presented prior to the stimulus. Post-exposure, chocolate consumption was measured, and the rest of the experiments were performed as before.

Furthermore, in the previous studies with TCDD, there was a conspicuous correspondence between ED50 values for the induction of hepatic CYP1A1 and the avoidance behaviour among all three tested, differentially TCDD-sensitive rat strains (Lensu *et al.* 2011b). Since AHR signalling is by far the predominant positive regulator of *Cyp1a1* gene expression (Q. Ma 2001), the finding suggested a possibility of a causal relationship between AHR activation in the liver and the behavioural change (Lensu *et al.* 2011b). In theory, AHR-induced induction of xenobiotic metabolising enzymes might not only be an index of AHR activation, but also functionally related to the mode of action of this behavioural change, *e.g.* by generating a critical mediator metabolite. However, the avoidance response could also be dependent on the enhancement of metabolic enzyme activation more generally, and not specifically in relation to activation of the AHR.

The latter question was approached in I by testing the effect of TPD, a phenobarbital-type inducer of CAR, a nuclear receptor likewise heavily involved in xenobiotic metabolism, but largely through CYP2B enzymes (Pustylnyak *et al.* 2009). The experiment was conducted identically to the experiment with FICZ, BNF and BaP, but 10 mg/kg of TPD was administered (n = 12 in TPD group, 6 in controls).

Persistence of the effect in the absence of novel food (I). In I, the persistence of novel food avoidance was also explored when access to the novel food was restricted. In this experiment, the rats were first exposed to either FICZ (0.1 mg/kg) or BNF (60 mg/kg), and then allowed access to novel chocolate on two occasions: first for a mere 6 h directly post-exposure, and again after a two-week interval, when it was left in the cages. Each group consisted of six rats.

Effect of vagotomy on the avoidance response (unpublished). The acquisition of CTA requires the activation of several brain regions. It was hypothesised that the novel food avoidance behaviour seen with AHR agonists would

similarly necessitate the activation of neuronal pathways. In I, the results had suggested that the stomach or the upper gastrointestinal tract might be a possible initial key target tissue for the avoidance behaviour. Thus, as the sensory input from the digestive tract, especially from its oral end, is mainly transmitted to the brain via the vagus nerve, it was examined whether blocking these messages by vagotomy would have an effect on the avoidance behaviour.

To this end, a single dose of BNF (60 mg/kg) was administered to vagotomised male SD rats (n =6/group) and chocolate offered as a novel food item directly post-exposure.

4.10 Clinical chemistry analysis (II)

Following the subacute toxicity study, clinical chemistry analyses from serum were carried out at the Central Laboratory of the Department of Equine and Small Animal Medicine, University of Helsinki. Enzymatic methods were used for the determination of serum FFA (a.k.a. long-chain fatty acids [LCFA] or non-esterified fatty acids [NEFA]) (NEFA-C, Waco Chemicals GmbH, Neuss, Germany) and D-3-hydroxybutyrate (3-HB; RANBUT, Randox Laboratories Ltd. Crumlin, UK). The analyses were performed with an automatic chemistry analyser (KONE Pro Selective Chemistry Analyser, Thermo Fisher Scientific). The rest of the serum analytes [alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), total bilirubin, creatinine, glucose, triglyceride, cholesterol and urea] were analysed using the reagents and adaptations recommended by the manufacturer of the automatic chemistry analyser (Konelab 30i, Thermo Fisher Scientific).

4.11 Thyroxine level measurement (II)

Thyroxine (T4) levels were measured in serum samples collected at the termination of the subacute toxicity study. For this, the Rat Thyroxine T4 ELISA Kit (Cusabio Biotech Co. Ltd, Wuhan, China) was used according to the manufacturer's instructions.

4.12 Retinoid concentration measurement (II)

Retinoid concentrations from serum, liver and kidney samples from the subacute toxicity study were measured at Instituto de Bioingeniería, Universidad Miguel Hernández de Elche (Alicante, Spain). Briefly, the different retinoid forms, extracted from tissue homogenates or serum, were separated by HPLC and detected by UV at

340 nm for retinoic acid derivatives (Schmidt, *et al.* 2003a), and at 325 nm for retinol and retinyl palmitate (van der Ven, *et al.* 2009), *i.e.* the polar and apolar retinoid forms, respectively.

4.13 Histopathological analysis (II)

Following the subacute toxicity study, liver, spleen, kidney, lung and testis samples were prepared and analysed at the Finnish Centre for Laboratory Animal Pathology (FCLAP), University of Helsinki. Briefly, the samples were fixed in 4% buffered formalin, embedded in paraffin and sectioned at 4 µm thickness. Slides were stained with haematoxylin-eosin for histopathological analysis.

4.14 RNA isolation from tissue samples (I, II, unpublished)

RNA was extracted from tissue samples for reverse transcription real-time quantitative PCR (RT-qPCR). Briefly, total RNA was isolated from bead-milled (TissueLyser LT, Qiagen, Hilden, Germany) tissue samples using the Sigma GenElute™ Mammalian Total RNA Miniprep Kit according to the manufacturer's protocol (Sigma-Aldrich). RNA was then treated with Ambion® TURBO DNA-*free*™ DNase treatment and removal reagent (Life Technologies, Carlsbad CA, USA). The concentration of total RNA was measured with a Nanodrop UV Spectrophotometer (Thermo Fisher Scientific) and RNA purity verified by 260/280 and 260/230 nm ratios.

4.15 RT-qPCR analysis of AHR-battery genes (I, II, unpublished)

RT-qPCR was performed to determine the expression of selected AHR-battery xenobiotic metabolising enzyme genes and *Cyp2b1* mRNA levels. The AHR-battery genes are a set of genes mainly encoding xenobiotic metabolising enzymes that include *Cyp1a1, Cyp1a2, Nqo1* and *Ugt1a6* (Nebert *et al.* 2000). Of these, induction of the *Cyp1a1* gene, in particular, is employed as a biomarker of AHR activation, as its induction is fairly rapid and it is highly sensitive (Abraham *et al.* 1988).

Total RNA was reverse transcribed to complementary DNA (cDNA) at 50 °C for 1 h using M-MLV RT RNase H-Point Mutant (Promega, Fitchburg WI, USA). For each reaction (25 µl), 50 or 100 U of the enzyme and 400 or 800 ng of RNA (respectively) was used. Quantitative real-time PCR (qPCR) was performed using HOT FIREPol® EvaGreen® qPCR Mix Plus (no ROX; Solis Biodyne, Tartu, Estonia) on Rotor-Gene

3000 or Rotor-Gene Q instruments (Qiagen, Hilden, Germany). This was carried out by absolute quantification, using the diluted total cDNA amount for normalization (20 ng/reaction, assumed based on the original amount of RNA in RT reactions; information on target gene specific primers in II, Supplementary Table 1) (Bustin 2002, Tichopad *et al.* 2009). Each cDNA sample was run in a duplicate reaction to obtain technical replicates. No-template controls were included in each run to control for reagent contamination. Primer specificity was confirmed by melt curve analysis at the end of each run. If the RT-qPCR result was below the detection limit, a conservative approach was taken and the sample was given the value of the limit.

The primers (Supplementary Table 1 in II) were designed based on the published genome sequences of the target genes using the Primer3web 4.0.0 web interface (Koressaar and Remm 2007, Untergasser *et al.* 2012). Standard curves were constructed for each primer pair by preparing a 10-fold dilution series starting from known concentrations of isolated and purified target gene PCR products amplified from cDNA samples, using the same primers as for RT-qPCR.

4.16 Molecular docking analysis (III)

In silico molecular modelling analyses were performed at the Department of Earth and Environmental Sciences, University of Milano-Bicocca, Italy. Briefly, a three-dimensional structure of the rat AHR LBD previously obtained (Motto *et al.* 2011) by homology modelling using MODELLER (Webb and Sali 2016) was employed. The molecular structures of C1 and C3 were subjected to conformational analysis performed by *ab initio* Quantum Mechanical (QM) calculations, using Jaguar (Schrödinger Release 2016-4: Jaguar, LLC, NY USA). Molecular Docking calculations were performed using Glide extra precision (XP; Schrödinger Release 2016-4: Glide). To include part of the domain flexibility involved in ligand binding, different modelled conformations of the AHR LBD were used for docking (ensemble-docking technique). One binding geometry (pose) was obtained for each ligand in each modelled receptor conformation, and the most favourable pose was selected by calculating the binding free energies with the Prime MM-GBSA approach (Schrödinger Release 2016-4: Prime).

4.17 Genotyping of the AHR knockout rat line (unpublished)

As both AHRKO and WT rats were required for the experiments, heterozygote rats were used for breeding the AHRKO rat line and all pups genotyped. For genotyping, genomic DNA (gDNA) was extracted from ear punches using hot sodium hydroxide and tris (HotSHOT) (Truett *et al.* 2000). The target gene was then amplified by

conventional PCR and DNA bands imaged under UV light after running the samples on ethidium bromide-stained 1–1.5% (w/v) agarose gels.

Briefly, ear punch samples were lysed in 25 mM NaOH and 0.2 mM disodium EDTA solution (pH ~12) by heating for 25 min at +95 °C. The samples were then cooled on ice and the lysates neutralised with 40 mM Tris-HCl (pH ~5), vortexed and centrifuged lightly. Four microlitres of supernatant was used for each PCR reaction (total volume 20 µl). The PCR reactions were performed using either JumpStart Taq DNA Polymerase (Sigma-Aldrich) or Universe High-Fidelity Hot Start DNA Polymerase (Bimake.com, Houston TX, USA) with target-gene-specific primers (Table 4). The reactions were amplified in Axygen® MaxyGene™ II thermal cyclers (further information on the PCR reactions provided in Table 4). Subsequently, 4 µl of loading dye was added to each sample, and reactions were loaded on 20 x 20 cm gels and electrophoresed at 110 V for 1 h in 1 x TAE buffer. Finally, the gels were imaged under UV light.

Table 4. Further information on the PCR reactions in genotyping the AHRKO rat line.

Enzyme	JumpStart Taq DNA Polymerase	Universe High-Fidelity Hot Start DNA Polymerase
Forward primer	cgggtgtgtctgttaatggc	(Same)
Reverse primer	tcctctcttgtccactgagc	(Same)
Supplements in PCR reactions (including H_2O, buffer, dNTP, primers, enzyme, and DNA)	$MgCl_2$, DMSO	Bovine serum albumin
Amplification	95 °C for 1 min; 35 cycles: 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 1 min 45 s; followed by 72 °C for 5 min.	95 °C for 3 min; 35 cycles: 95 °C for 15 s, 57 °C for 20 s, and 72 °C for 40 s; followed by 72 °C for 5 min.

4.18 Statistical analysis (I–III, unpublished)

SPSS Statistics software was applied for statistical analysis, and the level of significance was set at $p < 0.05$, unless otherwise specified (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0 or 24.0 Armonk NY, USA).

Most statistical analyses were carried out by one-way analysis of variance (ANOVA) followed by pairwise comparisons with Dunnett's test, Student-Newman-Keuls or Duncan's new multiple range post-hoc tests. In case the data were not normally distributed and variances were non-homogenous (as assessed by Levene's test), values were log-transformed in order to restore homogeneity, and then re-analysed by one-way ANOVA. In addition, results were verified by re-analysing the data with Kruskal-Wallis non-parametric ANOVA, followed by the Dunn-Bonferroni

post-hoc multiple comparison test. If data were normally distributed but variances were non-homogenous, post-hoc testing was performed by Games-Howell. When only two groups were compared with each other, the Student's t-test for independent samples was used. If t-test requirements were severely violated, the results were verified with the Mann-Whitney U-test.

Mixed between/within subject ANOVAs were used to analyse the data for repeated measurements over time in I and II. For this purpose, the normal distribution of the data was verified using Shapiro-Wilk's test, equality of error variances and covariance matrices were assessed using Levene's and Box's tests, respectively, and the homogeneity of the variances of the differences between all combinations of levels of the within-subjects factor (sphericity) was assessed with Mauchly's test. For one-way ANOVAs, significant main effects were further analysed by pairwise comparisons using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons, and the Kruskal-Wallis H-test followed by multiple comparisons. For two-way ANOVAs, simple main effects were analysed by univariate ANOVA and the Tukey HSD post-hoc test. In Box's test, only p-values of < 0.001 were considered significant.

In II, retinoid concentrations were analysed using R software version 3.2.3 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria). Pairwise multiple comparisons between exposed and control means were performed using ANOVA and linear contrast tests. In addition, Box plots were used for verification of a normal distribution.

Furthermore, in II, the 24-h luminescence data from the *in vitro* CYP1A1 enzyme activity assay were analysed by two-way ANOVA. The significance level for the interaction term was set at $p < 0.001$ in the case of non-homogenous variances (assessed by Levene's test) and a slight deviation from a normal distribution in some datasets (assessed by Shapiro-Wilk's test). Simple main effects were assessed by multiple pairwise comparisons with Bonferroni's adjustment. In III, the 48-h dose–response data from the CYP1A1 activity assay were analysed in GraphPad Prism using nonlinear regression with four-parameter logistics (GraphPad Software, Inc., Prism 7 for Windows, Version 7.03, La Jolla, CA USA). The data from the micronucleus test in III were statistically assessed by the Cochran-Armitage test for trend in proportions.

5 RESULTS

5.1 Characterisation of novel SAHRM effects

5.1.1 Toxicity *in vitro* (III)

LDH leakage assay. C1, C3 and TCDD were not cytotoxic in the LDH leakage assay. There was no dose response, and the maximum cytotoxicity caused by all three test compounds was only 5% at the concentrations tested, which was not statistically significant (Supplementary Figure 1 in III). In pairwise comparisons following one-way ANOVA ($p < 0.001$), the only group that differed from the others in a statistically significant manner ($p < 0.001$) was the positive control, 1% Triton X.

MTT reduction assay. A dose-dependent decrease in MTT reduction was observed for all three compounds, but at differing time points and concentrations (Figure 3 in III). At 6 h, multiple comparisons showed that for C1, the three highest concentrations (100, 500 and 1000 nM) differed statistically significantly from the vehicle controls, while C3 only had an effect at the highest concentration of 1000 nM. The MTT reduction capacities after 1000 nM exposures of C1 and C3 were down to 82% and 84% of controls, respectively. At 6 h, TCDD did not show a reducing effect at any of the concentrations tested (1–100 nM).

At 24 h, a significant decrease in MTT reduction was likewise only seen with C3 at the highest concentration. The results for C1 and TCDD were reversed compared with the 6-h time point. At 24 h, C1 did not induce a statistically significant effect at any concentration. With TCDD at 24 h, there was a statistically significant difference compared with the controls at the highest concentration tested, 100 nM, and the effect was more intense (down to 76%) than that of C1 and C3 at 100 nM or even 500 nM at either time point. For C1 and C3, the MTT reduction capacities at the highest concentrations were down to 82% and 77% of controls, respectively.

Interestingly, and unlike with TCDD, the effects of C1 and C3 did not intensify dramatically between 6 h and 24 h. Furthermore, between the 6- and 24-h time points, at concentrations of up to 100 nM, the MTT reduction capacity of cells treated with C1 and C3 appeared to have even slightly recovered, showing systematically slightly less of a reduction at 24 h than at 6 h.

Ames test. In the Ames test, C1 and C3 did not appear mutagenic in the dose range tested (1 nM–1 mM) in either of the strains used, TA100 and TA98. The number of revertants obtained with all tested concentrations of C1 and C3 was ≤ ~1-fold of the negative controls. The negative controls performed consistently, and the positive

controls also performed adequately overall, and all of them gave statistically significant positive results compared with the negative controls. However, in some cases, the indirect mutagen BaP gave rather low numbers of revertants with metabolic activation, indicating that the microsomal S9 mix did not work as efficiently as desired.

Some cytotoxicity was observed in the TA98 strain. The systematic dose responses were observed with metabolic activation by C1 at or above ~20 μM, and by C3 at or above ~70 μM, and without metabolic activation at the highest concentration of 1 mM by both C1 and C3. Furthermore, the plate concentration of 1 mM can be considered the maximum achievable, as both C1 and C3 already precipitated at this concentration when added to the master mix prior to plating.

Micronucleus test. As a minimum, a dose-dependent tendency towards increased frequency of micronuclei was observed by all three compounds (Table 1 in III). By the highest concentrations tested, C1 (1000 nM) and TCDD (100 nM), elicited fold-increases of 1.82 and 1.58 over negative controls, respectively. However, the Cochran-Armitage test of trend did not quite show statistical significance for C1 and TCDD ($p \geq 0.068$). C3 induced a maximal fold-increase of 2.67 by the highest dose of 1000 nM, and showed a statistically significant linear trend ($p=0.001$), with the proportion of cells containing micronuclei increasing as a function of concentration. The positive control mitomycin c (500 nM) induced a fold-increase of 10.44 in frequency of micronuclei.

5.1.2 Acute and subacute toxicity in SD rats (II)

Clinical signs of toxicity. No clinical signs of toxicity were seen during the acute toxicity experiment with either of the test compounds, or at any dose level tested. The same applied in the subacute toxicity experiment after 5-day dosing at high dose levels, except for peculiar, transient hyperaemia of the ear pinnae observed in both test compound groups (Supplementary Fig. 1 in II). This change appeared on the first day after the end of the dosing regimen and persisted for 3–4 days.

Contrary to the characteristic wasting syndrome of TCDD, BW gain tended to be only marginally decelerated in both experiments (Fig. 3 and Supplementary Table 2 in II). In the acute toxicity study, at 7 days post-exposure, a slightly delayed BW gain trend reached statistical significance ($p < 0.05$) only for the high-dose C4 group compared with the controls. In the subacute toxicity study, two-way mixed ANOVA revealed a statistically significant interaction in BW gain between treatment and time ($F[4,28] = 3.647$; $p = 0.016$; partial $\eta 2 = 0.343$), and subsequent univariate ANOVAs (followed by the Tukey HSD tests) at the three time points showed that the BW gain of C2 at 9 days (4.7%) was lower than that of the controls (10.1%; $p = 0.049$).

Organ weights. Both relative and absolute thymus weights exhibited a decreasing trend in the acute toxicity study, with a statistically significant ($p < 0.05$) relative weight loss of 30% in both C2 and C4 high-dose groups compared with the controls (Supplementary Table 2 in II). This effect was confirmed in the subacute toxicity study, where the relative weights were about 40% lower in both groups than in the control group (40% for C2, and 36% for the C4 group; one-way ANOVA, $p < 0.001$; Fig. 5). In the other organs sampled (liver, kidneys, spleen and testes), there were no statistically significant changes among groups.

Clinical chemistry. Clinical chemistry variables from serum were only analysed following the subacute toxicity study. The only marked alteration was a reduction of triglycerides by C2 (44%, $p = 0.02$; Fig. 7 in II). C4 had a similar effect, but the 30% decrease caused by it did not reach statistical significance. In addition, there was a statistically significant increase of 86% in the level of 3-HB by C4 ($p = 0.045$). A similar increase of 58% by C2 was not statistically significant.

Thyroxine level measurement. There were no statistically significant differences in thyroxine (T4) levels among the groups ($p = 0.426$; Supplementary Table 4 in II).

Retinoid analysis. Analysis of polar and apolar retinoid concentrations in the serum, liver and kidney was performed for the control, C2 and C4 groups after the subacute toxicity experiment. Overall changes in retinoid concentrations are presented in Table 5. The magnitudes of these effects were largely comparable between C2 and C4 (Table 2 in II). For most retinoid forms analysed, the changes recorded were slightly more pronounced in the C2 vs C4 group, in line with the higher administered dose of this compound. Two exceptions to this rule were provided by renal retinyl palmitate and serum 13-cis-retinoic acid concentrations, which were clearly more affected by C4 than C2.

Table 5. Statistically significant mean changes in retinoid concentrations by C2 (100 mg/kg/day) and C4 (75 mg/kg/day) in the subacute toxicity study (II), presented as fold changes over controls. P < 0.05, one-way ANOVA followed by linear contrast test.

Retinoid	Serum		Liver		Kidney	
	C2	C4	C2	C4	C2	C4
All-*trans* retinoic acid	–	–	1.3	–	1.3	–
Retinol	1.3	1.2	–	–	1.3	1.3
Retinyl palmitate	0.5	0.6	0.6	0.7	2.4	3.3
9-*cis*-4-oxo-13,14-dihydro-retinoic acid	0.4	0.6	0.1	0.1	–	–
13-*cis*-retinoic acid	0.5	0.4	–	–	nd	nd
4-hydroxy-all-*trans*-retinoic acid	nd	nd	0.3	0.3	nd	nd

– = statistically not significant
nd = not detected

Histopathology. Histopathology was not examined in the acute toxicity study, whereas in the subacute toxicity study, the liver, spleen, kidney, lung and testis were investigated. The only, very mild reaction was observed in the liver, where both C2 and C4 administration induced minimal hepatic extramedullary myeloid haematopoiesis (EMH; Fig. 6 in II) (Thoolen *et al.* 2010). This was observed in 3/5 C2-treated and in 4/5 C4-treated animals; none was present in controls.

5.1.3 Changes in the expression of AHR-battery genes (I–III)

CYP1A1 enzyme activity *in vitro* (II, III). C1 and C3 were tested *in vitro* in the H4IIE rat hepatoma cell line for their CYP1A1 enzyme induction potential, which was compared with that of TCDD. Exposure times were 24 h in II and 48 h in III.

In II, all of the compounds showed a statistically significant induction of CYP1A1 (ANOVA, $p < 0.005$), which increased in a dose-dependent fashion (Fig. 8 in II). Apart from the lowest dose level of 1 nM, each concentration of the compounds increased CYP1A1 induction in a statistically significant manner when compared with the control group.

In III, both C1 and C3 were likewise very effective and potent activators of CYP1A1 (Figure 4 in III). The potencies of all three compounds were similar: compared with the controls, C1 and C3 induced CYP1A1 statistically significantly at concentrations of 100 pM and above ($p \leq 0.009$ for C1 and $p \leq 0.013$ for C3), and TCDD from 500 pM on ($p \leq 0.008$). However, TCDD was the most potent inducer of the three. At 500 pM, the induction by TCDD was already more intense than by C1 and C3, attaining a 50-fold difference compared with the controls, while for C1 and C3, the respective fold changes were 10 and 20. The EC50 values for C1, C3 and TCDD were 24.1, 35.9 and 1.0 nM, respectively. The induction potential of TCDD peaked at 50 nM and appeared

to level off after that. Interestingly, compared with TCDD, both C1 and C3 attained an equivalent or even higher maximum induction of CYP1A1 at the highest concentration tested (1000 nM). Furthermore, it appears that the maximum response with C1 and C3 may not have been reached at 1000 nM, and therefore C3, and possibly also C1, have even higher efficacies than TCDD.

Furthermore, the ability of the selective AHR antagonist CH-223191 to block CYP1A1 induction by C1 and TCDD was tested with exposure times of 24 h and 48 h. At a concentration of 100 nM, CH-223191 was able to completely block the effect of 1 nM C1 and TCDD at both time points (Figure 5 in III).

***Cyp1a1* gene induction *in vivo* (I, II).** In I, there was a clear and statistically significant induction of hepatic *Cyp1a1* induction by single doses of C2 at all three dose levels tested ($p < 0.05$; Table 6). Even the lowest tested dose of 4 mg/kg induced *Cyp1a1* expression by 1700-fold at 28 h after exposure, a strong early response compared with the 350-fold increase brought about by a high, lethal dose of 0.1 mg/kg TCDD in L-E rats at 24 h in a previous study (Lindén *et al.* 2014).

However, while the response induced by TCDD intensifies during some days following exposure, the same did not apply for C2. After single doses of 20 and 100 mg/kg, hepatic *Cyp1a1* induction fold changes at 48 h and 72 h, respectively, were down to a ~100-fold increase compared with controls (Table 6). Likewise, in II, after 5-day repeated dosing at high dose levels, followed by 5 days of recovery before sampling, *Cyp1a1* induction by C2 and C4 was less pronounced than that at day 10 following a single dose of TCDD.

In addition to hepatic *Cyp1a1* induction, there was substantial *Cyp1a1* induction by C2 in all of the other tissues examined in II, and, apart from the testis, also by C4 ($p < 0.05$; Fig. 4 in II).

Table 6. Statistically significant hepatic inductions of selected AHR-battery genes by C2, C4 and TCDD, expressed as fold changes over controls. C2 and C4 were administered to SD rats for 5 consecutive days, unless otherwise specified. TCDD was administered once to L-E rats[1]. P < 0.05, one-way ANOVA/Student-Newman-Keuls or Student's t-test.

Gene	Sampling time (days post-exposure)	C2 100 mg/kg/day (*differing dosing*)	C4 75 mg/kg/day	TCDD[2] 100 µg/kg once
Cyp1a1	1	1700 *(4 mg/kg once)*	na	350
	2	100 *(20 mg/kg once)*	na	na
	3	130 *(100 mg/kg once)*	na	na
	10	370	140	1100
Cyp1a2	10	5	2	8
Cyp1b1	10	5	–	1600
Ahrr	10	6	3	230
Nqo1	10	3	–	50
Tiparp	10	–	–	25
Ugt1	10	1.4	–	7

na = not analysed

[1] Comparison between SD and L-E strains is considered justified, as the AHR-mediated induction of xenobiotic-metabolising enzymes is exhibited in the same fashion by all rat strains (M. A. Franc *et al.* 2008).

[2] The samples for the TCDD groups were from a study by Lindén *et al.* (2014), where TCDD-sensitive L-E rats were exposed to a single *ig* dose of 100 µg/kg TCDD, and necropsied at 10 days. The cDNA for these samples had been reverse-transcribed previously, but qPCR was performed with the same primers and in the same conditions as for C2 and C4 for comparison.

Induction of other selected AHR-battery genes *in vivo* (II, III). In addition to *Cyp1a1*, hepatic mRNA abundances of *Cyp1a2*, *Cyp1b1*, *Cyp2b1*, *Ahrr*, *Nqo1*, *Tiparp* and *Ugt1a* were determined in the repeated C2 and C4 exposure experiment (II). Overall, the inductions observed after C2/C4 exposures were much less pronounced than after exposure to TCDD (Table 6). The most conspicuous differences in the induction profiles of TCDD and C2/C4 were discernible in *Cyp1b1*, *Ahrr*, *Nqo1* and *Tiparp*, all of which were markedly induced by TCDD but feebly, if at all, by C2/C4.

5.1.4 Molecular docking analysis (III)

Simulation of ligand binding by molecular docking to the homology model of the rat AHR LBD predicted that the binding geometries as well as the protein–ligand

interactions of both C1 and C3 are similar to those of TCDD (Figure 6 in III). In fact, both compounds show mostly planar conformations in the docked poses, like TCDD, and occupy the central region of the ligand binding cavity (Figures 6b and 6c in III), similarly to what has been predicted for TCDD (Figure 6a in III) in the high-affinity mouse and rat AHRs (Motto *et al.* 2011). Most of the ligand-protein interactions that stabilize the C1 and C3 complexes are the same for the two ligands and cause similar binding affinities for the AHR. Stabilization is mainly due to hydrophobic interactions with residues within the binding cavity and to a hydrogen-bond with a central glutamine residue.

5.2 Novel food avoidance behaviour

5.2.1 Ability of AHR agonists to induce novel food avoidance (I)

All of the tested AHR agonists, FICZ, BNF and BaP, induced practically total abstinence from the consumption of chocolate (Figure 9). However, the duration of the effect varied: ~1 day for FICZ, 2–3 days for BNF and 3–4 days for BaP. The duration of the effect was assessed based on behavioural observation; the limit for fading of the avoidance was set at ~3 g of daily chocolate consumption, as 3 g was about half of the amount the controls consumed in the first 24 h post-exposure, and also roughly the amount after which consumption started increasing quite rapidly.

Similarly, C2 also induced total avoidance of the novel food at all the tested dose levels (Figure 9). At 100 mg/kg, the effect persisted for 2–3 days. The 4 and 20 mg/kg dose groups were only monitored for up to 24 and 48 h, respectively, until which the effect persisted. The animals in these groups were euthanized and sampled before the avoidance had passed to ensure that activation of AHR could be verified.

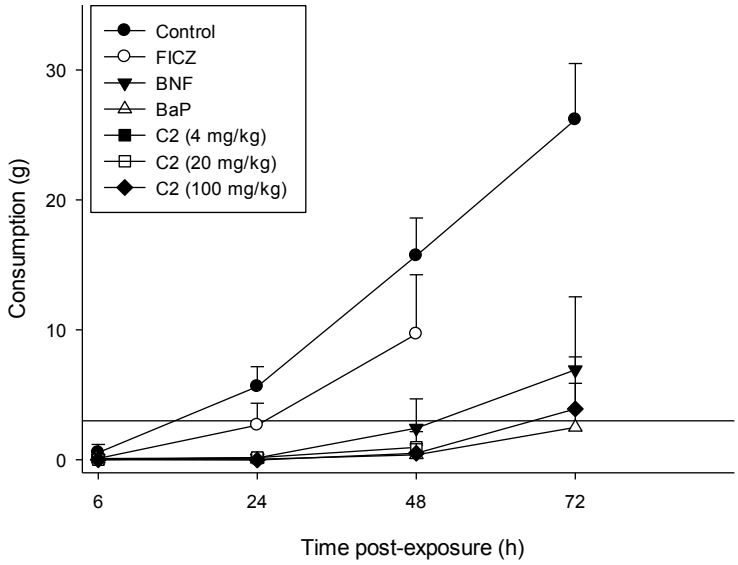


Figure 9. Cumulative consumption of chocolate after single doses of FICZ (0.1 mg/kg), BNF (60 mg/kg), BaP (150 mg/kg), C2 (4, 20, and 100 mg/kg) or the vehicle (mean + SD; n = 6 in all groups, except 12 in controls, both individual experiments combined). The reference line marks the consumption of 3 g, which was considered the limit value for the end of the avoidance phase. Chocolate consumption in the C2 4 mg/kg and 20 mg/kg groups was measured for only 24 and 48 h, respectively, in order to verify *Cyp1a1* induction.

5.2.2 Dependence of the response on the AHR (I, unpublished)

In I, the CAR activator TPD caused a robust and sustained hepatic induction of *Cyp2b1* expression (about 1000-fold; Table 1 in I), but in contrast to the results obtained with the AHR agonists tested, it did not influence chocolate consumption at all. By 24 h post-exposure, the TPD-exposed rats consumed 6.8 g (± 2.2 g) of chocolate, whereas the corresponding consumption in the control group was 6.2 g (± 3.0 g; Fig. 3 in I). Furthermore, TPD did not induce the genes of the AHR battery examined, apart from a tiny (3.3-fold) enhancement of hepatic *Cyp1a1* expression (Table 1 in I). Interestingly, there was one outlier rat in the TPD group that consumed practically no chocolate (0.3 g) during the 24 h of monitoring, exhibiting avoidance comparable to that in the BNF and BaP groups. RT-qPCR analysis revealed that while this individual's *Cyp2b1* induction in the liver and duodenum was similar to that in the rest of the TPD group (Table 1 in I), *Cyp1a1* was induced 300-fold in the stomach.

Furthermore, in I, both AHR-antagonists, CH-223191 and GNF351, failed to alleviate FICZ-induced novel food avoidance behaviour. It is noteworthy, however,

that both antagonists, administered separately *ig* as single doses (13 mg/kg of CH-223191 or 5 mg/kg of GNF-351), also failed to modify *Cyp1a1* gene induction caused by FICZ. Similarly, a single dose of CH-223191 failed to modify both *Cyp1a1* induction and avoidance behaviour by C2.

Moreover, in an unpublished experiment, AHRKO and littermate WT SD rats were employed for novel food avoidance response studies. The AHRKO genotype of this rat line was verified as AHR deficient by examining hepatic *Cyp1a1* expression levels following single BNF exposures (60 mg/kg, *ig*). As expected, the *Cyp1a1* gene was not expressed in the AHRKO rats or induced in them by BNF, contrary to the WT lineage (Figure 10).

In the first experiment conducted with the AHRKO rat line, the study design was identical to that used previously, and novel chocolate was placed in the cages directly after exposures. However, both genotypes behaved differently compared to the Harlan SD rat line used in the previous experiments, as the controls exhibited a considerably slower onset of chocolate consumption than before. By 24 h, the WT and KO control group consumption averages were 1.6 ± 2.4 g and 1.9 ± 2.9 g, respectively. Despite this, by 48 h, it appeared evident that unlike in WT rats, BNF did not induce avoidance behaviour in the KO rats, although the low number of animals (n = 3/group) constrained the interpretation of this result. The WT control and BNF groups had consumed on average 13.3 ± 5.9 g and 1.2 ± 0.9 g of chocolate, respectively, while in the KO rats, the respective control and BNF group chocolate consumptions were 5.8 ± 6.7 g and 9.3 ± 3.8 g.

In the second experiment with the AHRKO rat line, chocolate was placed in each cage for ~24 h prior to exposures. Chocolate consumption during the first 24 h before exposures was 9.2 ± 1.8 g and 7.9 ± 1.7 g in the WT and KO lines, respectively. After exposures, as hypothesised, BNF failed to influence chocolate intake in the KOs; both the control and BNF groups consumed on average 6.3–6.6 g by 24 h ($p = 0.875$; Figure 10). In contrast, in the WT lineage, BNF-treated rats exhibited clear chocolate avoidance, while the controls did not (respective 24-h consumptions: 1.3 vs 8.3 g, $p = 0.011$; Figure 10).

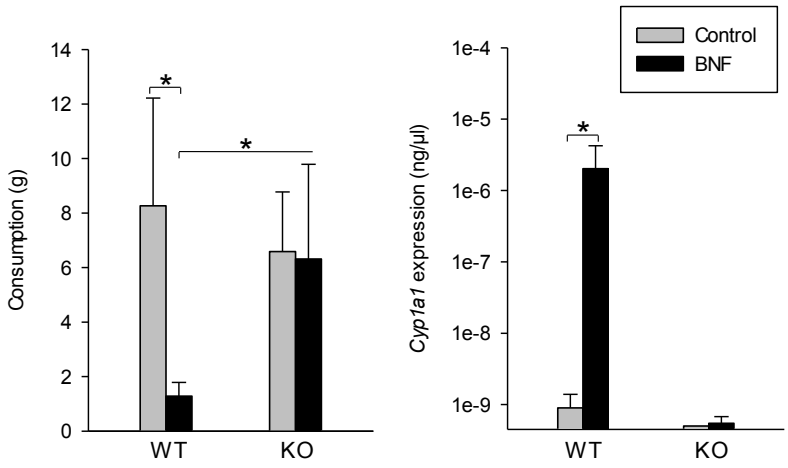


Figure 10. **Left panel:** 24-h cumulative consumption of chocolate (mean + SD; n = 6–7) after a single dose of BNF (60 mg/kg) or vehicle in WT and KO rats. The groups differing from each other in a statistically significant fashion are marked with an asterisk ($p < 0.05$). **Right panel:** Hepatic *Cyp1a1* expression (mean + SD, logarithmic scale) within both genotypes, confirming that the KO line is AHR deficient. The BNF groups differing in a statistically significant manner from their controls, within each genotype, are marked with an asterisk ($p < 0.05$).

5.2.3 Persistence of the effect in the absence of novel food (I)

Similarly to previous experiments, during the initial 6-h period post-exposure, the AHR-agonist-treated rats nibbled only minute amounts of chocolate, if any (Fig. 4A in I), after which it was removed from the cages. At the re-introduction two weeks later, both AHR activators still provoked the avoidance of chocolate, which resembled that of the first encounter in the earlier studies. While controls reached the set limit value of 3 g chocolate intake by approximately 6 h, for rats treated with FICZ or BNF, this took 6–24 h or 24–48 h, respectively (Fig. 4A in I).

5.2.4 Effect of vagotomy on the avoidance response (unpublished)

Vagotomy did not prevent the emergence of the novel food avoidance behaviour in the AHR-agonist-treated group. By 48 h post-exposure, rats in the control group consumed an average of 8.6 ± 2.2 g of chocolate, while rats in the BNF group exhibited clear avoidance, consuming only 0.3 ± 0.2 g of chocolate ($p = 0.02$).

6 DISCUSSION

The AHR is a transcription factor involved in numerous functions within organisms, and imparts both beneficial physiological and harmful adverse effects. The mechanisms are highly diverse and complex, and currently incompletely understood. In addition to conveying the toxicity of environmental contaminants, such as dioxins, it appears that inappropriate modulation of the AHR has a role in the pathogenesis of several illnesses. Therefore, it is also an intriguing target for pharmacological research. So far, the AHR has mostly been considered as a potential target for treatment of immunological diseases and cancer. However, it is quite possible that we are not yet aware of all its functions, and the AHR could therefore also be employed as a target for the treatment of other types of diseases and conditions.

As lead compound candidates for drug development, SAHRMs are particularly interesting, as they only produce subsets of AHR-mediated effects. Therefore, they could conceivably be optimised with the aim of producing beneficial effects of AHR modulation, omitting toxicity.

Furthermore, SAHRMs could be valuable tools in further elucidating the so far incompetently understood, multifaceted physiological roles and effects of the AHR, and the underlying molecular mechanisms and pathways. Increasing information on them could also help us understand the aetiology and pathogenesis of adverse outcomes that we are currently only attempting to characterise, such as the effects that the increasing amounts of chemicals in our environment may have on our health.

This thesis research examined the toxicity of two novel SAHRMs, intended as potential drug compounds. It also further characterised a peculiar novel food avoidance behaviour in rats, which had previously been described following TCDD exposure.

6.1 Effects of the novel SAHRMs

Previously, very limited experimental information was available about the novel pro-drugs C2 and C4 and their active metabolites, the SAHRMs C1 and C3. The few earlier studies had primarily aimed at exploring their pharmacological potential, which had proven promising. In the EAE model in rats, a total dose of 4 mg/kg C2 (*sc*) had efficiently prevented EAE development (Pettersson 2012). It had also had an ameliorating effect in the dextran sulfate sodium-induced colitis model in mice, at a dose of 1 mg/kg (*po*; unpublished data).

In this study, selected toxicological properties of these compounds were examined. Their effectiveness and potency in activating the AHR were also

characterised and compared with TCDD. Furthermore, the binding of the three compounds to the rat AHR was explored *in silico*.

6.1.1 AHR-activation potential and binding to the AHR (I–III)

To study the potency and efficacy of C1 and C3 as AHR activators *in vitro*, the metabolically active H4IIE rat hepatoma cell line was employed and the novel compounds compared with TCDD. The AHR-activating potential was assessed as induction of the xenobiotic-metabolising enzyme CYP1A1, as it is sensitive biomarker of AHR activation (Abraham *et al.* 1988).

Screening of the compounds in II revealed that both novel SAHRMs, C1 and C3, induced responses quite similar to that of TCDD. Closer scrutiny in III reinforced the results, and showed that both compounds are similar to TCDD in their efficacy and potency of AHR activation. The latter experiments also confirmed that C3 appears to be the somewhat more effective compound of the two. However, it should be noted that only a continuous hepatoma cell line was used in these experiments. Including normal cells would have strengthened the investigation, and possibly given more robust information, as their physiology better reflects that of healthy organisms.

The *in vitro* results were furthermore supported by the *in vivo* data on *Cyp1a1* gene induction in I, which demonstrated that C2 is a highly effective inducer of the *Cyp1a1* gene following single doses at several dose levels (Table 6). However, while the response with TCDD intensifies during some days following exposure, the same does not apply for C2. Therefore, it appears that unlike TCDD, C2 and C4 are rapidly metabolised in rats, with an elimination half-life within the range of hours to a couple of days. However, the potent *Cyp1a1*-induction by C2 at one day post-exposure implies that also *in vivo*, the inherent effectiveness of C2 may be, at minimum, comparable to that of TCDD.

In addition to *Cyp1a1*, both C2 and C4 also induced several other AHR-battery genes of xenobiotic metabolism, but the induction profiles were distinct from that of TCDD (Table 6), indeed suggesting selective modulation.

Furthermore, in III, the ability of C1 and C3 to bind the rat AHR was studied by *in silico* methods, in comparison with TCDD. The simulations revealed that the binding geometries of C1 and C3 are similar to those of TCDD, and that both ligands adopt mostly planar conformations in the docked poses. Interestingly, most of the residues involved in the stabilization of C1 and C3 belong to the group of highly conserved residues lining the binding cavities of several mammalian AHRs, and are necessary for optimal TCDD binding (Motto *et al.* 2011, Pandini *et al.* 2009). Therefore, the computational results support the hypothesis that these novel SAHRMs effectively bind to the AHR and act as its agonists.

This conclusion is supported by the *in vitro* finding in III that the AHR antagonist CH-223191, reported to only be a selective antagonist of dioxin-like AHR activators

(S. -. Kim *et al.* 2006, Zhao *et al.* 2010), was able to block CYP1A1 induction by C1 as efficiently as that of TCDD. This further supports the view that at least C1 binds to the LBD of the AHR in a manner similar to that of TCDD.

6.1.2 Toxicity *in vitro* and *in vivo* (II, III)

***In vitro* toxicity.** *In vitro* toxicity testing was employed to provide initial predictions of possible toxic effects *in vivo*. In this study, C1 and C3 were examined for their potential to induce cytotoxicity in the LDH leakage assay, their effect on metabolic activity in the MTT reduction assay, and their genotoxicity in the Ames test and in the micronucleus assay.

In III, the novel SAHRMs, like TCDD, were not cytotoxic, as assessed by the LDH leakage assay. However, while being a robust method and widely used, the LDH assay only detects cytotoxicity following damage to the cell membrane, as LDH is released from the cytosol. Therefore, it does not identify compounds that reduce viability through other pathways. Thus, C1, C3 and TCDD were also tested using the MTT reduction assay. In the MTT reduction assay, dose-dependent reductions in metabolic activities were observed by all three compounds. However, the effects of C1 and C3 did not intensify dramatically between 6 h and 24 h, in contrast to the result following TCDD exposure. Furthermore, the cells treated with C1 and C3 appeared to have even slightly recovered at concentrations up 100 nM by 24 h. By this time point, TCDD reduced metabolic activity at a ≥ 10-fold lower concentration of 100 nM than C1 or C3. Therefore, considering that the EC50 values for C1 and C3 were found to be 24 and 36 nM, respectively, and that metabolic activity was down to only ~80% of vehicle control activity even with the highest concentrations of 1000 nM tested, C1 and C3 do not appear cytotoxic in the MTT reduction assay. Furthermore, these results support the view that C1 and C3 are rapidly metabolised to inactive products, unlike TCDD.

The *Salmonella* Typhimurium strains used in the Ames test were TA98 and TA100, which contain the *hisD3052* and *hisG46* mutations, respectively (Maron and Ames 1983). The TA98 strain primarily detects mutagens that cause frameshift mutations, while the TA100 preferentially detects mutagens causing base-pair substitutions. Only C1 and C3 were tested here, as it has been previously shown that TCDD is not mutagenic (Thornton *et al.* 2001). Similarly to TCDD, neither C1 nor C3 were mutagenic in the Ames test. However, at doses of >20 μM for C1 and at >70 μM for C3, the compounds appeared cytotoxic in the TA98 strain. Nevertheless, the highest tested plate concentrations of 1 mM can be considered the maximum achievable due to precipitation on the plates at that dose level. Therefore, and despite some limitations in the efficiency of the S9 mix, the negative result in the Ames test can be considered reliable for both compounds, particularly in the TA100 strain. It thus appears that in the Ames test, C1 and C3 do not induce base-pair substitutions and are unlikely to induce frameshift mutations.

Yet, in the micronucleus assay, C3 induced a statistically significant, increasing trend in number of micronuclei, while C1 and TCDD showed a tendency for statistical significance. The significance of the positive effect by C3 is unclear, however, particularly considering the small magnitude of the fold-increase vs. the positive control mitomycin c, and the similar result obtained with TCDD. This is due to TCDD being known to not be a genotoxicant *in vivo* (Huff *et al.* 1991, Meyne *et al.* 1985, Nebert *et al.* 2004), even though particularly *in vitro*, it has occasionally appeared positive at high concentrations in genotoxicity tests, including a micronucleus test in rat hepatocytes (Turkez *et al.* 2014). Therefore, in order to conclude whether the effect seen here with C3 is relevant, an *in vivo* micronucleus test may be necessary.

Only one cell line was employed in the *in vitro* experiments, apart from the Ames test where two strains were used. The H4IIE was selected for its exceptional metabolic activity (Fujimura *et al.* 2012) and because it has been shown to be very responsive to dioxins (Bradlaw and Casterline Jr. 1979, Sawyer and Safe 1982). Nevertheless, the investigation would have been strengthened by repeating the experiments with other cell lines, as different lines typically respond, at least to some extent, differentially under the same conditions. Including a normal cell line would have furthermore strengthened the information about the *in vitro* effects of these compounds. However, the methods used are generally considered robust for the purpose of screening compounds, and overall it can be concluded that apart from the equivocal result of the micronucleus test, the results from *in vitro* testing do not raise concern for toxicity *in vivo*.

***In vivo* toxicity.** *In vivo*, C2 and C4 were studied for toxicity in male SD rats in II, first after acute dosing and then after 5-day repeated dosing. TCDD was not tested here, but results from the literature were used to compare the effects.

The single dose experiment confirmed that the acute toxicity of the compounds was low, even at the highest doses practically achievable, which were 100 mg/kg for C2 and 75 mg/kg for C4. During the experiment, there were no apparent clinical signs of toxicity, and the compounds were therefore deemed suitable for repeated administration. However, thymus size was significantly diminished by single high doses of both compounds, and a tendency towards dampened growth was observed with the high dose of C4. Nevertheless, because the numbers of animals used at each dose level in this experiment were low (n = 1–3), the results were regarded as indicative.

In the repeated-dosing study, C2 and C4 were tested at only one dose level each due to a shortage of available resources. The undesirable consequence of this limitation was that the study design did not allow investigating the dose-dependency of the observed effects. The doses used were 100 mg/kg/day for C2 and 75 mg/kg/day for C4 on five consecutive days, followed by a 5-day monitoring period. The exposure period was short, but as the doses were high, the study can be expected to have

revealed the short-term toxic potential of the test compounds, particularly for any sensitive endpoints.

The rats tolerated the treatments well overall also following repeated dosing, even though the compounds were not without effects. Interestingly, the profiles of C2 and C4 appeared distinct from that of TCDD, supporting the view that these compounds are truly selective; both C2 and C4 lacked some of the major characteristic toxic effects of TCDD, shared some with it, but also exhibited a small number of adverse effects not seen with TCDD, all presented in Table 7.

Of the characteristic toxic effects of TCDD that were lacking with C2 and C4 in this study, lethality and wasting syndrome are perhaps the most notable. Considering that C2, C4, and TCDD also appear to be similarly effective as inducers of *Cyp1a1 in vivo*, it is noteworthy that each C2 and C4 dose of 75–100 mg/kg/day, administered on five days, was 1500–2000 times higher than the TCDD LD50 of ~50 µg/kg in the SD strain (M. Franc *et al.* 2001). However, only a slight downward tendency was recorded in BWs following C2 and C4 administration. While, as already established, C2 and C4 appear to be metabolised much more rapidly than TCDD, it does not appear plausible that merely a difference in kinetics could explain such a drastic difference in the toxic effects.

Nevertheless, two characteristic adverse effects of TCDD were also observed with C2 and C4, although somewhat less pronounced: thymic atrophy and changes in tissue retinoid (vitamin A) concentrations (Fletcher *et al.* 2001, Gupta *et al.* 1973, M. W. Harris *et al.* 1973). The occurrence of thymic atrophy is particularly interesting, as it is one of the most consistent and uniform effects of TCDD across mammalian species (Pohjanvirta and Tuomisto 1994). However, the toxicological relevance of this effect in adults, rodents or humans, is unclear, as thymus function is mostly relevant during the prenatal period, and physiological atrophy of the thymus ensues by adolescence. Furthermore, the thymus does not appear to be involved in dioxin-induced immunotoxicity (Kerkvliet and Brauner 1987).

As to retinoid homeostasis, no firm conclusions can yet be drawn on the full extent to which the alterations induced by C2 and C4 resemble those of TCDD, as there are not enough data in the literature on the effects of TCDD on several of the retinoic acid derivatives in the tissues that were analysed in this study. However, some of the hepatic and renal changes induced by C2 and C4 were reminiscent of those seen after short-term TCDD exposure in male rats (Hoegberg *et al.* 2003, Nilsson *et al.* 2000, C. K. Schmidt *et al.* 2003), while others seen in C2- and C4-treated rats were not typical effects of TCDD. Moreover, high TCDD doses have been reported to induce changes that remained unaltered following C2 or C4 exposure. More information is therefore needed on these changes to evaluate their significance.

Moreover, some characteristic adverse effects common to TCDD exposure were not considered in these experiments due to technical reasons, and thus information on the effects of C2 and C4 on these is, for the time being, lacking completely. These include further effects on the endocrine system, such as changes in testosterone,

insulin or melatonin levels, changes in the degree of oxidative stress in various tissues, bone and tooth lesions, immuno- and developmental toxicity, and carcinogenicity. The occurrence of all of these should naturally be tested in the future.

Altogether, C2 and C4 brought about only a subset of the studied response spectrum previously reported with TCDD (Table 7), and all these effects belong to the type I category (Pohjanvirta *et al.* 2011). The effects in this category are robust to structural variations in the AHR transactivation domain, and thereby represent more generic AHR-mediated impacts. Whether any of type II responses, such as wasting syndrome, would manifest during longer exposure periods or if higher doses of C2 or C4 could be administered is a matter of speculation and should be studied further. However, the slight downward tendency recorded in BWs in the present study might suggest that the existence of early alterations in the adverse outcome pathway that ultimately culminates in the wasting syndrome cannot be entirely ruled out.

Table 7. Selected type I and II responses typical after a single high dose of TCDD (Pohjanvirta and Tuomisto 1994, Pohjanvirta *et al.* 2011, Viluksela *et al.* 1999), and their occurrence in SD rats after repeated 5-day dosing with C2 (100 mg/kg/day) and C4 (75 mg/kg/day), followed by a 5-day monitoring period. Furthermore, effects of C2 and C4 not seen after TCDD exposure are presented.

Type	Effect	TCDD	C2	C4
I	Induction of *Cyp1a1* gene	+	+	+
	Induction of all studied AHR-battery genes	+	–	–
	Thymus atrophy	+	+	+
	Changes in retinoid homeostasis	+	+	+
	Hypercholesterolaemia	+	–	–
	Reduced plasma thyroxine levels	+	–	–
	Novel food avoidance behaviour	+	+	na
II	Lethality	+	–	–
	Wasting syndrome	+	–	–
	Grave liver lesions	+	–	–
	Testis lesions	+	–	–
	Hypoglycaemia	+	–	–
	Elevated plasma FFA levels	+	–	–
–	Ear hyperaemia	–	+	+
–	Minimal hepatic EMH	–	+	+
–	Reduction of serum triglycerides	–	+	(+)
–	Increase of serum 3-HB	–	(+)	+

+ = present; – = lacking; na = not assessed, (+) = present, but not statistically significant

In addition to the two adverse effects typical of TCDD, C2 and C4 also induced effects that have not been reported with TCDD, presented in Table 7. Of these, ear hyperaemia was perplexing and unexpected, and should be further examined in future studies. EMH refers to haematopoiesis occurring outside of the medullary spaces of the bone marrow (Johns and Christopher 2012, C. Kim 2010). It is mostly considered to lack serious clinical or diagnostic implications, and can occur without any obvious underlying cause or consequences. Therefore, and as the hepatic EMH seen in this study was minimal, it may not imply any relevant toxicity. However, its possible recurrence should not be overlooked in future studies with these compounds, as EMH can occur secondary to, for instance, local hypoxia, bone marrow insufficiency and myelotoxicity (Chiu *et al.* 2015). As to the observed changes in clinical chemistry, the decrease in triglycerides and elevation of 3-HB point to enhanced β-oxidation and lowered *de novo* fatty acid biosynthesis in the liver, which could in fact be interpreted as beneficial effects.

Most AHR ligands bind to the receptor with moderate or low affinities compared to TCDD, and most of them also have lower abilities to induce CYP1A1 (Safe *et al.* 2011). The novel SAHRMs studied here, however, were predicted *in silico* to possess binding affinities similar to that of TCDD, and they are almost similarly effective inducers of CYP1A1 *in vitro* and *Cyp1a1 in vivo*. Based on *in silico* modelling, they also appear to bind to the rat AHR in a manner similar to that of TCDD. However, the compounds show markedly less toxicity *in vivo* in rats, both after acute and repeated 5-day dosing. Nevertheless, they were not completely without adverse effects, although none of them appeared grave. Importantly, considering the effectiveness of these novel SAHRMs, the doses used in this study can be regarded to have been very high. Thus, as they are apparently truly selective in their effects, these SAHRMs appear interesting candidates for therapeutic uses. They may also have wider implications for AHR research, and could be valuable tools in further elucidating the multifaceted physiological roles of the AHR and the underlying molecular mechanisms.

In the future, it would be important to further characterise the toxicological effects of these compounds. In particular, because they appear to be metabolised rapidly but are potent and effective AHR activators, it would be crucial to investigate whether prolonged exposure to C2 and C4 *in vivo* would elicit more pronounced toxicity than seen here. In addition, examining their kinetics and elimination rate in rats would give important information, as well as a comparative metabolism study in rat and human normal hepatocytes. Furthermore, it will be vital to study these compounds in representative human cell cultures and micro tissues, as these results are limited to rats. As previously discussed, different AHR agonists commonly exert varying effects among species, complicating extrapolation between species. However, as these compounds elicited only type I responses of TCDD, previously demonstrated to be largely indifferent to structural variation at the transactivation domain of the AHR in TCDD-treated rats (Pohjanvirta *et al.* 2011), pre-clinical data with these compounds

could arguably also be reasonably relevant to human hazard assessment, unless type II effects also emerge during prolonged exposure or at higher doses. Moreover, if future binding modelling would reveal that C1 and C3 are also bound to the human AHR in a manner similar to that of TCDD, it is conceivable that their toxic effects would be less pronounced in humans than in rats, as humans appear to be less sensitive to TCDD than most rat strains. This outcome would naturally also be likely to improve the prospects of employing these SAHRMs as safe or tolerable drug compounds. Finally, it would be highly interesting to analyse their effects on the whole transcriptome by RNA sequencing in order to thoroughly assess their similarities and differences with TCDD.

6.2 The AHR and novel food avoidance (I, unpublished)

Previously, it was discovered that concurrent exposure to TCDD and presentation of novel food items caused rats and mice to exhibit strong and persistent dislike towards novel foods (Lensu *et al.* 2011a, Lensu *et al.* 2011b, J. T. Tuomisto *et al.* 2000). Moreover, there appeared to be a correlation between ED50 values for the induction of hepatic CYP1A1 and novel food avoidance. The critical participation of the AHR in this bizarre effect appeared likely, as virtually all biological effects of TCDD require AHR modulation, and the avoidance was not seen in AHRKO mice. Furthermore, since AHR signalling is the predominant up-regulator of *Cyp1a1* gene expression (Q. Ma 2001), the finding suggested a possibility of a causal relationship between hepatic AHR activation and the behavioural change (Lensu *et al.* 2011b).

However, interpretation of the results in KO mice had been somewhat hampered by the unexpected finding that consumption of the novel food was higher in the TCDD-treated than in the vehicle-treated group (Lensu *et al.* 2011b). Furthermore, the early experiments did not explore whether this effect was only related to TCDD, more generally to the induction of xenobiotic metabolism, or specifically to activation of the AHR. Therefore, in this study, it was set out to further investigate the involvement of the AHR in novel food avoidance behaviour in rats, in which this response had been found to be more pronounced than in mice following TCDD exposure (Lensu *et al.* 2011b).

6.2.1 Dependence of the response on the AHR

In I, it was established that the induction of novel food avoidance behaviour was not limited to TCDD, but also occurred with all of the other AHR agonists tested, FICZ, BNF and BaP. FICZ, BNF and BaP were each only studied at one dose level, and therefore dose response could not be considered. Interestingly, the SAHRM C2 was also shown to induce novel food avoidance. With C2, it appears likely that the lowest

effect level would be considerably beneath the lowest tested dose of 4 mg/kg, based on the strong response. The high potency of C2 probably explains why no dose-response was observed, despite of testing at three dose levels.

Furthermore, in two currently unpublished experiments, the novel food avoidance behaviour was not seen in AHRKO rats after BNF exposure, while it was evident in littermate WT rats. However, two AHR antagonists tested, CH-223191 and GNF351, were unable to alleviate FICZ- and C2-induced avoidance behaviour, but this was probably due to insufficient doses and/or pharmacokinetic reasons, as they appeared to be generally unable to antagonise AHR function in this experiment, revealed by the clear induction of hepatic *Cyp1a1*. Moreover, in contrast to the outcome with the AHR agonists, strong phenobarbital-like induction of xenobiotic metabolising enzyme activity following exposure to a CAR agonist, TPD, did not elicit the avoidance behaviour, as shown in I. Therefore, novel food avoidance behaviour appears to be specifically and exclusively dependent on AHR activation and induced by both exogenous and endogenous AHR agonists.

Interestingly, there were differences in the duration of the avoidance behaviour induced by the different AHR activators tested (Figure 9). Earlier, TCDD induced avoidance that persisted much longer than with the other compounds, lasting 14 days with constant *ad libitum* access to the novel food. The shortest effect in this study was seen with FICZ (~1 day), while the effects of BNF, BaP and C2 lasted for 2–3 days.

These differences are probably due to at least three reasons. Firstly, the doses of the test compounds were not comparable in potency, as they were selected based on previous literature data to provide a reliable induction of drug-metabolizing enzymes in the liver. In particular, the 150 mg/kg dose used for BaP was quite high, and at 100 mg/kg *ig* BaP has been reported to cause adverse effects in rats, including a transient reduction in motor activity (Knuckles *et al.* 2001, Saunders *et al.* 2002). Secondly, the test compound mixtures differed due to technical reasons, and BNF and BaP were administered as suspensions, while FICZ and C2 were solutions. This could have slightly affected the durations of the effects induced by BNF and BaP, as absorption from suspensions may be prolonged compared with solutions. However, the effect of 100 mg/kg C2, dosed as a solution, also persisted for ~3 days. Thirdly, and conceivably most importantly, it appears likely that the durations reflect differing elimination half-lives of the compounds tested, even though neither this study nor available literature data allow its precise verification. However, TCDD has a biological half-life of approximately 3 weeks in rats (H. J. Geyer *et al.* 2002, Piper *et al.* 1973, Pohjanvirta *et al.* 1990), while FICZ is known to be metabolized very rapidly in mammals (Wincent *et al.* 2012). Kinetic data are lacking for C2 and BNF, but BaP or its metabolites may persist for several days in certain tissues (Ramesh *et al.* 2001). For C2, based on the results in I–III, metabolism also appears to be clearly more rapid than that of TCDD.

Further characterisation of the novel food avoidance behaviour in I revealed that when rats initially had merely 6 h of access to chocolate upon exposure to the AHR

activators FICZ or BNF, the avoidance response was still clearly present two weeks later when chocolate was offered again. This could suggest that AHR activation is essential in triggering the avoidance, but might no longer be required during its maintenance. On the other hand, in the second encounter, chocolate avoidance did subside at a faster rate than in the first one. Thus, at this point, the results concerning the role of the AHR in maintaining the avoidance of novel foods are inconclusive.

Furthermore, although both nausea and gastrointestinal malaise are subjective experiences and thus poorly amenable to studying in laboratory animals, earlier results with TCDD as well as the results in I suggest that malaise is not a critical prerequisite for AHR-mediated novel food avoidance behaviour in rats. For both BaP (Saunders *et al.* 2002) and TCDD (Pohjanvirta *et al.* 1994), doses higher than those employed in these and previous novel food avoidance studies have been reported not to cause malaise in rats. Additionally, all the three C2 doses (single 4, 20 and 100 mg/kg) eliciting a comparable, reliable and substantial novel food avoidance response were far below those causing overt toxicity.

6.2.2 The AHR and novel food avoidance: a connection to CTA?

The results of this study were not conclusive as to the primary behavioural phenomenon underlying the AHR-agonist-induced avoidance of novel foods and its possible connection to CTA. This was mainly due to technical reasons limiting the experimental design. However, the design used in the second, currently unpublished experiment with the AHRKO rat line did allow exploring this question, as for CTA to develop, the association of the taste (or odour) of the CS with the avoidance stimulus (US) is essential. Furthermore, in classical CTA designs the CS is typically presented prior to the US. Here, even when chocolate (CS) was first offered for ~24 h prior to exposure (US), and both WT and KO rats readily consumed it then, avoidance behaviour was evident after exposure in the WT BNF group, but non-existent in the KO BNF group (Figure 10). This indicates that CTA may well be involved. There are also several previous results, reviewed in chapter 2.4, pointing towards CTA, although other prior results suggest the involvement of neophobia.

The acquisition of CTA is due to neural processing by the brain, which integrates the CS with the consequences of the US (Bermudez-Rattoni 2014). The signal may be transmitted to the brain by neural afferents or humorally, but the physiological site where it originates can be difficult to identify, especially in the absence of an obvious symptom, such as malaise. In I, the results from one outlier rat had suggested that AHR activation in the stomach or upper gastrointestinal tract might be a possible initial key target for the avoidance behaviour. However, based on a third, currently unpublished experiment, the vagus nerve, which transmits sensory input from the digestive tract to the brain, does not seem to be involved in conveying this behavioural effect to the nervous system. Nevertheless, the digestive tract remains a credible

peripheral site for the novel food avoidance effect, and signals from there could be transmitted via other routes.

Astrocytes, an abundant cell type of the central nervous system (CNS), are interesting regarding this matter. They are specialised glial cells that have important and diverse roles in health and disease, and have been shown to regulate metabolism, modulate neuronal transmission and be involved in CNS development and repair (Rothhammer *et al.* 2016, Sofroniew and Vinters 2010). In addition, astrocytes appear to be involved in CNS autoimmunity, for instance in the pathogenesis of MS and EAE. Furthermore, they have been shown to limit CNS inflammation following exposure to AHR agonists originating from dietary tryptophan and metabolised by the gut microbiota (Rothhammer *et al.* 2016). Therefore, it is conceivable that astrocytes could be involved in conveying also other effects induced by AHR agonists in the gastrointestinal tract, including the signals resulting in novel food avoidance.

As discussed earlier, in addition to its several physiological functions, AHR signalling mediates toxic effects resulting from exposure to xenobiotics. Therefore, it is conceivable that it has been an evolutionary advantage to develop a system that detects and appropriately responds to even small changes in its activity. Furthermore, considering that many bHLH-PAS proteins are sensors responding to environmental and cellular signals (Furness *et al.* 2007, Gasiewicz and Henry 2011, Gu *et al.* 2000), it would not be surprising if one important physiological function of the AHR were to act as a sensitive sensor producing an early protective response to potentially harmful ingested foods. The ability of the AHR to bind compounds with various structures would be highly beneficial in this task, as it is valuable for the survival of organisms to consume any nutrition available. Moreover, as briefly discussed under *Dietary AHR modulators* in chapter 2.2.1, many dietary AHR modulators are frequently present in the GI tract, and it is plausible that they could have additive, potent local effects. Finally, it is highly interesting that in previous studies with TCDD, a particularly strong and persistent avoidance response developed towards chocolate over cheese, sucrose and saccharin solutions (Lensu *et al.* 2011a, J. T. Tuomisto *et al.* 2000). There may be several explanations for this, but an intriguing possibility are flavonoids and other polyphenols, which are present in cocoa and chocolate in high quantities (Katz *et al.* 2011, Lamuela-Raventós *et al.* 2005, Vinson *et al.* 1999), and might be able to potentiate the effect of the administered AHR agonist.

In addition to providing formal confirmation that AHR signalling is a necessary and sufficient mediator of AHR-agonist-induced novel food avoidance behaviour in rats, this study raises several questions concerning the novel food avoidance behaviour triggered by AHR activation for future research. First, it would be intriguing to confirm whether there is a relationship between it and CTA. Most of the known inducers of CTA, including the “gold standard” lithium chloride (LiCl), have not been reported to exhibit AHR activation. However, very few of them have been examined for this property, and it is also possible that they could affect the AHR

through non-canonical signalling. For example, LiCl is a known GSK3b-inhibitor and thus a Wnt signalling activator, and Wnt signalling in turn can modify AHR activity (Grimes and Jope 2001, Klein and Melton 1996, Schneider *et al.* 2014). Examining the effect of LiCl in the AHRKO rat model would thus be of great interest. Second, the critical site(s) of AHR activity for the avoidance response and the mechanism by which the signal is then delivered to the CNS call for further scrutiny.

Finally, the possible repercussions of these findings for humans remain to be established. CTA is a common clinical problem in human medicine with chemo- and radiotherapy, and may also emerge in other nausea-inducing conditions, such as food poisoning and motion sickness (Arwas *et al.* 1989, Bernstein 1985, Scalera 2002). It has even been utilized in alcoholism treatment as "emetic therapy" (Elkins 1991). In theory, it could be possible to target the AHR in treatment of these conditions or, for instance, obesity. However, it is perhaps more likely that the relevance for humans related to this intriguing effect would come through increased understanding of the physiological functions of the AHR in the gastrointestinal tract. Indeed, information on other such functions are already emerging. Those encompass particularly impacts on intestinal inflammation, microbiota, energy balance, obesity and related steatosis (Hubbard *et al.* 2017, Moyer *et al.* 2016, Moyer *et al.* 2017, Pohjanvirta 2017, L. Zhang *et al.* 2015). Therefore, the role of the AHR in feeding behaviour might also have broader implications in the future.

Particularly, the microbiota of the gastrointestinal tract may prove to be important in relation to the novel food avoidance behaviour. The gut microbiota and host immune system are known to interact and to regulate each other (C. H. Kim 2018). For instance, host genes have been shown to have an impact on the composition and function of the microbiota of the gastrointestinal tract, resulting in alterations in the production of microbial metabolites and intestinal inflammation (Lamas *et al.* 2016). Furthermore, one of the mechanisms by which the microbiota regulate the host metabolism and immune system is by production of metabolites (C. H. Kim 2018). As briefly discussed in chapter 2.2.2, several endogenous tryptophan metabolites are AHR agonists and appear to have important roles in the mammalian gut immune homeostasis. Interestingly, also different types of microorganisms in the gut have been shown to metabolise tryptophan into AHR-active compounds, and immune cells are known to express the AHR (Jin, Lee, Sridharan *et al.* 2014, C. H. Kim 2018, Wille *et al.* 2001, Zelante *et al.* 2013). Such metabolites have, for instance, been shown to balance mucosal reactivity in mice (Zelante *et al.* 2013). Therefore, a microbiota-AHR axis in the gut may prove to be important in the immunity of vertebrates (Rothhammer *et al.* 2016, Zelante *et al.* 2013). Concerning novel food avoidance, this could also be of significance regarding microbial contamination of food and formation of toxic or infectious material. The possibly resulting food avoidance behaviour would protect organisms from further adverse effects, offering them an evolutionary advantage.

7 CONCLUSIONS

1) The active SAHRMs C1 and C3 are not cytotoxic or mutagenic *in vitro*, but they are very potent and effective activators of the AHR, in fact comparable to TCDD. Based on *in silico* modelling and validation of the analysis by *in vitro* experimentation, both C1 and C3 bind to the LBD in the rat AHR in a manner very similar to that of TCDD, occupying the central region of the ligand-binding cavity.
2) The prodrug SAHRMs C2 and C4 are also potent *in vivo* activators of the AHR in rats. However, they lack some major characteristic toxic effects of TCDD. In addition, overall, their observed effect profiles appear distinct from that of TCDD, and pharmacokinetics is likely to play a role in this.
3) Both novel SAHRMs are promising compounds that may have potential as pharmaceutical molecules, and as valuable tools in further elucidating the multifaceted physiological roles of the AHR and the underlying molecular mechanisms.
4) Induction of novel food avoidance in rats is not limited to TCDD, but other AHR agonists also induce it, including endogenous and exogenous compounds and the SAHRM C2. The effect appears to be specifically and exclusively dependent on AHR activation. CTA may be involved, although further studies are warranted to investigate its involvement.

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